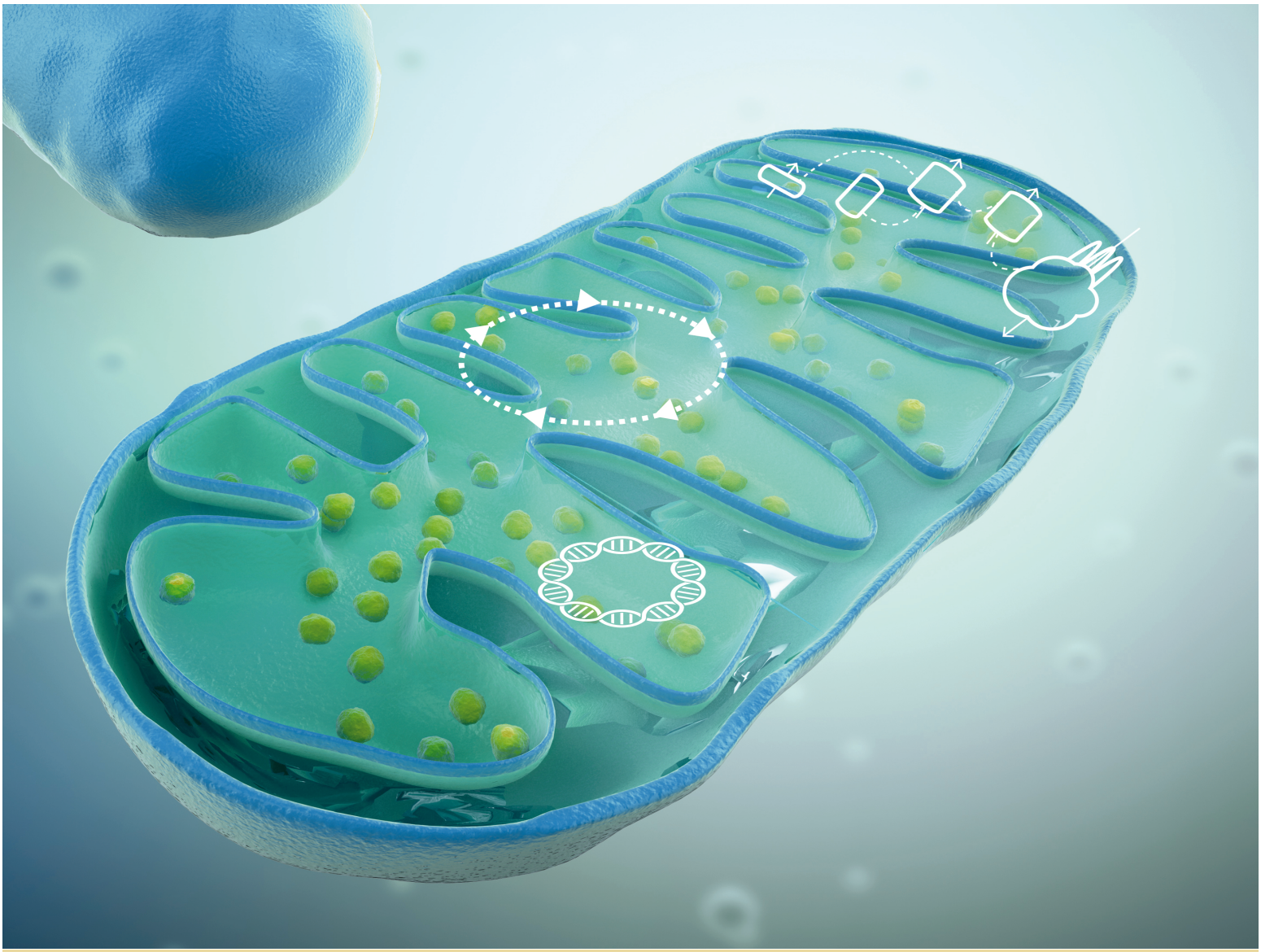

Filipa Sofia Libório Carvalho

Clarification of the Mitochondrial Role in the Cardiotoxicity of Doxorubicin Using a Whole Heart Perfusion System - Impact of Different Doxorubicin Treatment Regimens

Tese de Doutoramento em Biociências, especialidade Toxicologia orientada pelo Doutor Paulo Oliveira e pelo Doutor Rui de Carvalho e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Setembro/2014

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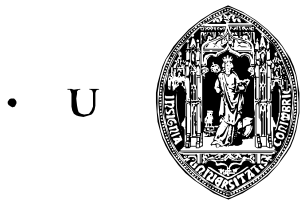
Universidade de Coimbra

Clarification of the Mitochondrial Role in the Cardiotoxicity of Doxorubicin Using a Whole Heart Perfusion System - Impact of Different Doxorubicin Treatment Regimens



UNIVERSIDADE DE COIMBRA

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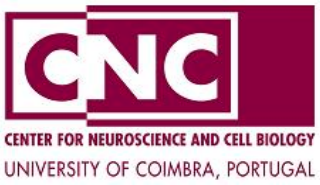

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"Out of the mountain of despair, a stone of hope"

Martin Luther King

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Abbreviations

ADP- Adenosine diphosphate;

AIF- Apoptosis Inducing Factor;

AMPK- Adenosine monophosphate-activated protein kinase;

ANT-Adenine nucleotide translocator;

ATP- Adenosine triphosphate;

Casp- Caspase;

CK- Creatine kinase;

CO_2- Carbon dioxide;

Cr- Creatine;

CsA- Cyclosporin A;

CypD- Cyclophilin D;

Cyt c- Cytochrome c;

DOX- Doxorubicin;

FELASA- Federation of European Laboratory Animal Science Associations

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase;

GG- Galactose plus glutamine;

GLUT- Glucose transporter;

GTP- Guanosine triphosphate;

Hif-1α- Hypoxia-inducible factor 1-alpha;

Hsp90- Heat shock protein 90;

IMM- Inner mitochondrial membrane;

IMS-Interspace mitochondrial membrane;

Iodo- Iodoacetate;

JNK- c-Jun N-terminal kinase;

KCN- Potassium cyanide;

LDH- Lactate dehydrogenase;

LDHA- Lactate dehydrogenase A isoenzyme;

LVDP - Left ventricular developed pressure;

MAPK- Mitogen-activated protein kinase; ADP- Adenosine diphosphate;

MPT- Mitochondrial Permeability Transition;

MtCK -Mitochondrial CK isoenzymes;

mtDNA- mitochondrial DNA;

NADH- Nicotinamide adenine dinucleotide;

OM- Octanoate plus malate;

OMM- Outer mitochondrial membrane:

OXPHOS- Oxidative phosphorylation;

PCr- Phosphocreatine;

PDH- Pyruvate dehydrogenase;

PGC-1α- Peroxisome proliferator-activated receptor-gamma coactivator;

PiC- Phosphate carrier;

PKM 1/2- Pyruvate kinase;

RCR- Respiratory control ratio;

ROS- Reactive Oxygen Species;

Rot- Rotenone;

RPP- Rate pressure product;

SAL- Saline;

TC- Time Control;

TCA- Tricarboxylic acid cycle;

TFAM- Mitochondrial transcription factor A;

VDAC- Voltage dependent anionic channel;

$\Delta\psi m$- Mitochondrial transmembrane potential;

Abstract

Doxorubicin (DOX) is one of the most potent antineoplastic drugs. Although possessing a superior anti-cancer activity, a broader clinical use of DOX is limited by a dose-dependent, constant and cumulative cardiomyopathy involving deterioration of mitochondrial function.

Although acute effects of DOX treatment often disappear when treatment finishes, chronic effects often result in a persistent cardiotoxicity, including a progressive deterioration of mitochondrial metabolism, the development of cardiomyopathy and ultimately congestive heart failure. Important in the context of DOX-induced cardiotoxicity, mitochondrial disruption has been observed in different models. This alteration of mitochondrial function is often sub-clinical and is only manifested as cardiomyopathy when other factors are combined, including age or different types of cardiovascular stress. Also, metabolic alterations in the cardiac cell occur, which contribute to the ability of the heart to withstand increased workloads. Although mitochondrial disruption is an early and sensitive marker of DOX cardiotoxicity, how metabolic stress contributes to the development of cardiomyopathy remains to be determined. Because of this gap in knowledge, the objective of this work was to use of model of metabolic inhibition in perfused hearts from saline (SAL) and DOX-treated rats in order to identify metabolic alterations caused by an acute and sub-chronic DOX treatment.

Our assumption for this strategy is that a lower susceptibility to a determined inhibitor during perfusion, would be a sign of a more robust (i.e. more capacity) of the targeted pathway(s).

For the acute treatment protocol, sixteen week-old male Wistar rats were i.p. injected with 20mg/Kg DOX or 1mg/Kg 0.9%NaCl and sacrificed 24 hours later. For sub-chronic protocol, eight weeks-old male Wistar rats received seven weekly s.c. injections with DOX(2mg/Kg) or equivalent SAL solution and sacrificed one week after the last injection. Following the protocol treatments, animals were sacrificed and hearts were removed and perfused using a Langendorff apparatus with distinct cardiac substrates (glucose, galactose plus glutamine - GG or octanoate plus malate – OM). Glycolytic (iodoacetate) and oxidative phosphorylation (rotenone- Rot or cyanide- KCN) inhibitors were separately added to the different metabolic perfusion buffers, aiming to detect undercover mitochondrial defects in the DOX-treated group. In non-perfused hearts, or hearts perfused in the absence (time controls, TC) or presence of inhibitors, selected metabolic and mitochondrial proteins were semi-quantified by Western blotting, and mRNA levels were quantified by RT-PCR.

In the acute DOX treatment model, hearts perfused with glucose as substrate suffered a decline in the number of heart beat and rate pressure product (RPP) when iodoacetate was added, contrarily to Rot or KCN which had no effect. With GG, inhibitor titration decreased the heart rate, despite that the decrease in the RPP was more evident in SAL vs. DOX group with iodoacetate and KCN. Perfusion with OM resulted in decreased heart rate an RPP in the presence of the inhibitors, showing equal response between treatments. When glycolytic and mitochondrial proteins were semi-quantified by Western blotting, alterations in proteins involved in mitochondrial biogenesis and autophagy were observed in DOX hearts perfused with inhibitors. The data from the acute protocol study, appears to suggest that hearts from DOX-treated animals have improved function in the presence of

metabolic inhibitors, thus indicating that DOX triggers adaptations that allow the hearts to be less susceptible to mitochondrial and glycolytic inhibition.

In the sub-chronic model and using glucose as substrate, the DOX-treated group showed again a better tolerability to inhibitors than SAL. With GG, titration with iodoacetate caused a decrease in heart beat and on RPP in DOX group, when rot or KCN was added the number of heart beat and RPP remains identical between the two groups. Glycolytic and mitochondrial proteins semi-quantification suggested an impairment of autophagy in DOX perfused hearts perfused, more evident during GG perfusion. The presence of inhibitors in the perfusion also generally decreased the total amount of proteins detected by Western Blotting, although glycolytic proteins were increased when hearts were perfused with glucose, contrarily to GG perfusion.

The results suggest that sub-chronic DOX-treated rats suffered a metabolic remodeling which is based on stronger glycolytic fluxes to maintain contractility, although no overt mitochondrial defect was uncovered.

A surprising result is that regardless of the perfusion buffer used, no striking differences between SAL and DOX hearts in terms of hemodynamic parameters were found.

The present work suggests that metabolic remodeling during DOX acute and sub-chronic treatment maintains cardiac function in the treated animals. This remodeling is apparently based in a stronger contribution of glycolysis to overall metabolism. Data from protein amount analyzed suggest that DOX treatment in both models affect important regulators of autophagy, mitochondrial biogenesis as well as the adenine nucleotide translocator. The results also suggest that a longer treatment

protocol or resting period should also be tested in order to uncover more profound differences.

Resumo

A doxorubicina (DOX) é um dos fármacos antineoplásicos mais potentes. Apesar de possuir uma actividade anti-cancro superior, uma mais ampla utilização clínica da DOX é limitada por uma cardiomiopatia dose-dependente, constante e cumulativa que envolve a deterioração da função mitocondrial.

Embora os efeitos agudos do tratamento DOX normalmente desapareçam quando se conclui o tratamento, os efeitos crónicos resultam muitas vezes numa cardiotoxicidade persistente, incluindo a deterioração progressiva do metabolismo mitocondrial, o desenvolvimento de cardiomiopatia e por fim insuficiência cardíaca congestiva. Importante no contexto da cardiotoxicidade induzida pela DOX, a perturbação mitocondrial foi observada em diferentes modelos. Esta alteração da função mitocondrial é muitas vezes sub-clínica e só se manifesta como cardiomiopatia quando outros factores são combinados, incluindo a idade ou diferentes tipos de estresse cardiovascular. Além disso, ocorrem alterações metabólicas na célula cardíaca, o que contribui para a capacidade do coração resistir a maior necessidades. Embora a perturbação mitocondrial seja um marcador precoce e sensível de DOX cardiotoxicidade, permanece por determinar como o stress metabólico contribui para o desenvolvimento de cardiomiopatia. Devido a essa lacuna no conhecimento, o objetivo deste trabalho foi a utilização de um modelo de inibição metabólica em corações perfundidos de ratos tratados com uma solução salina (SAL) e ratos tratados com DOX, a fim de identificar alterações metabólicas causadas por um tratamento agudo e sub-crónico.

O nosso pressuposto para esta estratégia é que uma menor susceptibilidade a um determinado inibidor durante a perfusão, seria um sinal de uma forma mais robusta (ou seja, maior capacidade) da (s) via (s)-alvo (s).

Para o protocolo de tratamento agudo, ratos machos Wistar de 16 semanas de idade foram injectados i.p. com 20 mg / kg de DOX ou de 1 mg / kg a 0,9% de NaCl e sacrificados 24 horas mais tarde. Para o protocolo sub-crónico, ratos machos Wistar de oito semanas de idade, receberam sete injecções s.c. semanais com DOX (2 mg / kg) ou uma equivalente da solução SAL sendo sacrificados uma semana após a última injecção. Após o protocolo de tratamentos, os animais foram sacrificados e os corações foram perfundidos com um aparelho de Langendorff com substratos cardíacos distintos (glucose, galactose mais glutamina - GG ou octanoato mais malato - OM). Inibidores glicolíticos (iodoacetato) e inibidores da fosforilação oxidativa (Rot- rotenona ou KCN- cianeto) foram adicionados separadamente nos diferentes substratos metabólicos, com o objetivo de detectar defeitos mitocondriais no grupo tratado com DOX. Em corações não perfundidos, ou corações perfundidos na ausência (controlos de tempo, TC) ou na presença de inibidores, algumas proteínas metabólicas e proteínas mitocondriais foram semi-quantificadas por Western blotting, e os níveis de mRNA foram quantificados por RT-PCR.

No modelo de tratamento agudo DOX, corações perfundidos com glucose como substrato sofreram um declínio no número de batimentos cardíacos e produto da taxa de pressão (RPP), quando iodoacetato foi adicionado, ao contrário da Rot ou KCN, que não teve nenhum efeito. Com GG, a titulação com o inibidor diminuiu a frequência cardíaca, apesar de que a diminuição da RPP foi mais evidente no grupo SAL vs. DOX com iodoacetato e KCN. Perfusão com OM resultou em diminuição da

frequência cardíaca e do RPP na presença dos inibidores, mostrando uma resposta igual entre os tratamentos. Quando proteínas glicolíticas e mitocondriais foram semi-quantificadas por Western blotting, foram observadas alterações de proteínas envolvidas na biogênese mitocondrial e autofagia em corações DOX perfundidos com inibidores. Os dados do protocolo do estudo agudo, parecem sugerir que os corações provenientes de animais tratados com DOX na presença de inibidores, têm a função metabólica melhorada, indicando assim que a DOX desencadeia adaptações que permitem que os corações sejam menos susceptíveis à inibição mitocondrial e glicolítica.

No modelo sub-crónico e utilizando glucose como substrato, o grupo tratado com DOX mostrou novamente uma melhor tolerabilidade para inibidores que SAL. Com GG, a titulação com iodoacetato causou uma diminuição no batimento cardíaco e no RPP em grupo DOX, quando Rot ou KCN foi adicionado o número de batimentos cardíacos e RPP permaneceram idênticos entre os dois grupos. A semi-quantificação de proteínas glicolíticas e mitocondriais sugerem uma deficiência da autofagia em corações DOX perfundidos, mais evidente durante a perfusão com GG. A presença de inibidores da perfusão também reduziu a quantidade total de proteínas detectadas através de Western Blot, embora proteínas glicolíticas tenham aumentado quando os corações foram perfundidos com glucose, ao contrário da perfusão com GG.

Os resultados sugerem que ratos tratados sub-cronicamente com DOX sofreram uma remodelação metabólica, que é baseado em fluxos glicolíticos mais fortes para manter a contratilidade, embora nenhum defeito mitocondrial ostensivo tenha sido descoberto.

Um resultado surpreendente é que, independentemente do tampão de perfusão utilizado, não foram encontradas diferenças marcantes entre SAL e DOX corações em termos de parâmetros hemodinâmicos.

O presente trabalho sugere que existe uma remodelação metabólica durante o tratamento agudo e sub-crónico com DOX, que mantém a função cardíaca nos animais tratados. Esta remodelação é, aparentemente, baseada numa contribuição mais forte da glicólise para o metabolismo geral. Os resultados de quantidade de proteína analisados sugerem que o tratamento com DOX em ambos os modelos afectam importantes reguladores da autofagia e biogénese mitocondrial, bem como o translocador de nucleótidos de adenina. Os resultados também sugerem que um protocolo de tratamento mais longo ou com um período de repouso também devem ser testados a fim de descobrir diferenças mais profundas.

Short summary

Doxorubicin (DOX) is an anticancer anthracycline that presents a dose-dependent and cumulative cardiotoxicity involving deterioration of mitochondrial function. The impact of metabolic injury/remodeling on cardiac performance is still undefined.

The main goal of the work is to confirm our hypothesis that mitochondrial dysfunction is an early marker for DOX toxicity by pinpointing defects in the energy machinery in the hearts of DOX-treated rats.

Acute and sub-chronic DOX treatments were performed in Wistar rats, and hearts were perfused with different cardiac substrates, in the presence of mitochondrial and glycolytic inhibitors, aiming to detect undercover mitochondrial defects in DOX-treated group. Metabolic and mitochondrial proteins were semi-quantified by Western blotting, and mRNA analyzed by qRT-PCR.

Results of hemodynamic parameters suggest that in both acute and sub-chronic treatments, DOX hearts have improved function in the presence of metabolic inhibitors, indicating that DOX triggers adaptations that allow the hearts to be less susceptible to mitochondrial inhibition. Observing results from protein amount and mRNA in both treatments models, proteins related with autophagy, mitochondrial biogenesis and function suffered treatment-related alterations.

In conclusion, use of glycolytic pathway is favored in heart in the presence of DOX, however, cardiac defects incited by hidden mitochondria dysfunction was not detected.

Sumário resumido

A doxorubicina (DOX) é uma antraciclina anti-cancerígena que causa uma cardiotoxicidade cumulativa e dependente da dose que envolve a deterioração da função mitocondrial.

O principal objetivo deste trabalho é confirmar a nossa hipótese de que a disfunção mitocondrial é um marcador precoce de toxicidade da DOX através da identificação de defeitos na produção de energia no coração de ratos tratados com DOX.

Tratamentos agudos e sub-crónicos com DOX foram realizados em ratos Wistar, e os corações foram perfundidos com diferentes substratos cardíacos, usando inibidores específicos para a mitocondria e glicólise, com o objetivo de detectar defeitos mitocondriais no grupo tratado com DOX. Proteínas metabólicas e mitocondriais foram semi-quantificadas por Western blotting e mRNA analisados por qRT-PCR.

Os resultados dos parâmetros hemodinâmicos sugerem que em tratamentos agudos e sub-crónicos com DOX, os corações têm a função melhorada na presença de inibidores metabólicos, indicando que a DOX desencadeia adaptações que permitem que os corações sejam menos susceptíveis à inibição mitocondrial. Proteínas relacionadas com a autofagia, biogénese e função mitocondrial foram alteradas devido ao tratamento com DOX.

Em conclusão, a utilização da via glicolítica foi favorecida na presença de DOX, no entanto defeitos resultantes de disfunção mitocondrial não foram detectados.

Chapter 1: General Introduction

1. Mitochondria: Cell battery

Mitochondria are a cellular organelles identified by the first time by Rudolph Albert Von Kölliker in 1857. However, the final designation of mitochondria was proposed by Benda only in 1898 [1]. Over the next years, several studies emerged demonstrating that mitochondria have a central role in cellular energy production [2], as well as being involved in calcium homeostasis, progression of cell death and intermediate metabolism [3,4]. The number of mitochondria present in each cell greatly depends upon its metabolic requirements, and may range from hundreds to thousands. High metabolic tissues, such as skeletal muscle, have more mitochondrial mass than tissues with lower metabolic requirements [5].

1.1 Mitochondria structure and energetic function

Mitochondria are formed by two membranes, an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), with the intermembrane space (IMS) located between both membranes. The internal mitochondrial milieu surrounded by the IMM is called matrix [3]. The OMM is characterized by the presence of specific transporters, such as the voltage dependent anionic channel (VDAC) [6], among others, while the IMM has high content of the phospholipid cardiolipin which plays an important role in the maintenance of mitochondrial function, through greatly reducing the permeability of the membrane to protons and by regulating mitochondrial proteins including cytochrome c oxidase and the adenine nucleotide translocator (ANT) [7]. Importantly, the mitochondrial matrix contains several copies of mtDNA and the machinery needed for its replication,

transcription and translation, which confer to this organelle the unique capacity for self-replication [8].

Mitochondria are responsible for the synthesis of approximately 95% of the ATP needed by the cell, with this energy being obtained by a process called oxidative phosphorylation (OXPHOS) [9]. Hence, the maintenance of mitochondrial homeostasis is an important task for the cell and is tightly regulated through gene expression patterns, with impairments in gene transcription often associated with mitochondrial dysfunction and disease states (Fig. 1).

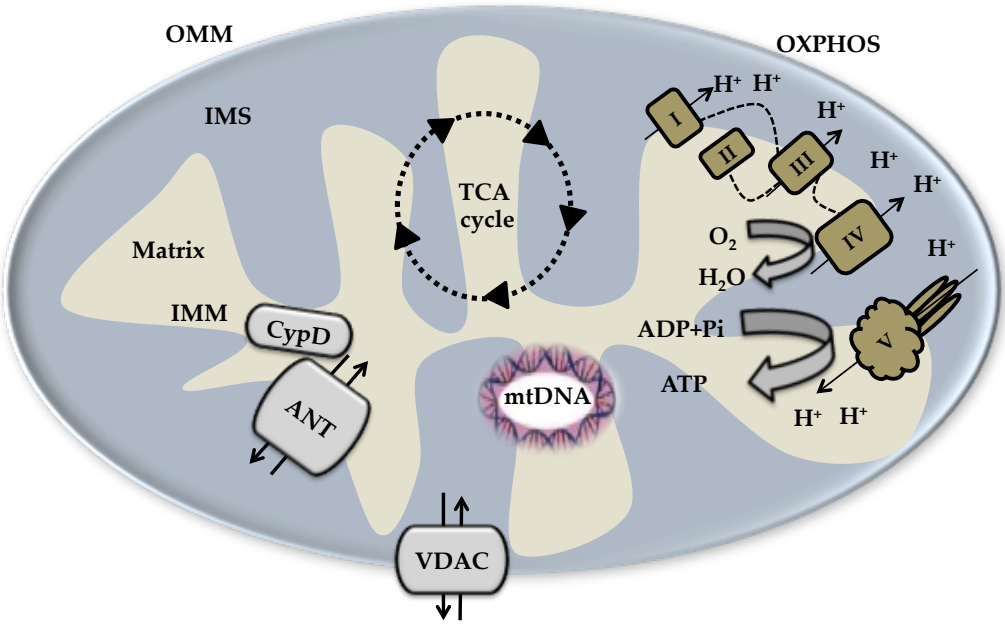

Figure 1. *Mitochondria as the center of cellular bioenergetics.*
The figure represents different functions of mitochondria, including oxidative phosphorylation (OXPHOS) that pumps protons generating a proton electrochemical gradient (Δψm), used by the ATP synthase (complex V) to produce adenosine triphosphate (ATP), the energetic fuel to cells. The tricarboxylic acid (TCA) cycle supplies nicotinamide adenine dinucleotide (NADH) and succinate to the respiratory chain. Proteins such as the voltage dependent anionic channel (VDAC), adenine nucleotide translocator (ANT) or cyclophilin D (CypD) are essential to the maintenance of mitochondrial homeostasis preventing cell death.
Abbreviations: *IMM- inner mitochondrial membrane; OMM- outer mitochondrial membrane: IMS-interspace mitochondrial membrane; mtDNA- mitochondrial DNA*

Several metabolic pathways take place in the mitochondria, including OXPHOS, the tricarboxylic acid (TCA) cycle and fatty acid β-oxidation [7]. We next focus on glycolysis, the TCA cycle and fatty acid β-oxidation.

Glycolysis is the first step in aerobic ATP production from glucose. Glucose is imported to the cell by glucose transporters (GLUTs) and metabolized through sequential reactions to generate pyruvate [10]. Pyruvate is converted to acetyl-CoA in the mitochondrial matrix by pyruvate dehydrogenase (PDH), and then enters the TCA cycle in mitochondria. This metabolic pathway consists in sequential chemical reactions associated with the production of guanosine triphosphate (GTP), carbon dioxide (**CO_2**), succinate, flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH), the latter two supplying OXPHOS [3]. Under conditions of normal nutrient supply, most glucose is converted to pyruvate, which enters mitochondria. Under low oxygen conditions, pyruvate is diverted to produce lactate through anaerobic glycolysis. Although anaerobic glycolysis is far less efficient, it provides critical intermediate metabolites necessary to maintain macromolecular biosynthesis [11].

Fatty acids are a very important source of energy, assuming a pivotal role in the maintenance of energetic homeostasis and consisting in the degradation of fatty acid by a catabolic process know as fatty acid β-oxidation. This process occurs mainly in the mitochondrial matrix, with long-chain fatty acids being first transported from the cytoplasm to mitochondria, where they undergo β-oxidation to generate acetyl-CoA, used for ATP production via the TCA cycle and OXPHOS. Indeed, in conditions where carbohydrates are less abundant, such as during prolonged fasting, exercise or

metabolic stress, fatty acid oxidation assumes a central role as energy source, therefore contributing for the maintenance of cellular homeostasis [12] (Fig. 2)

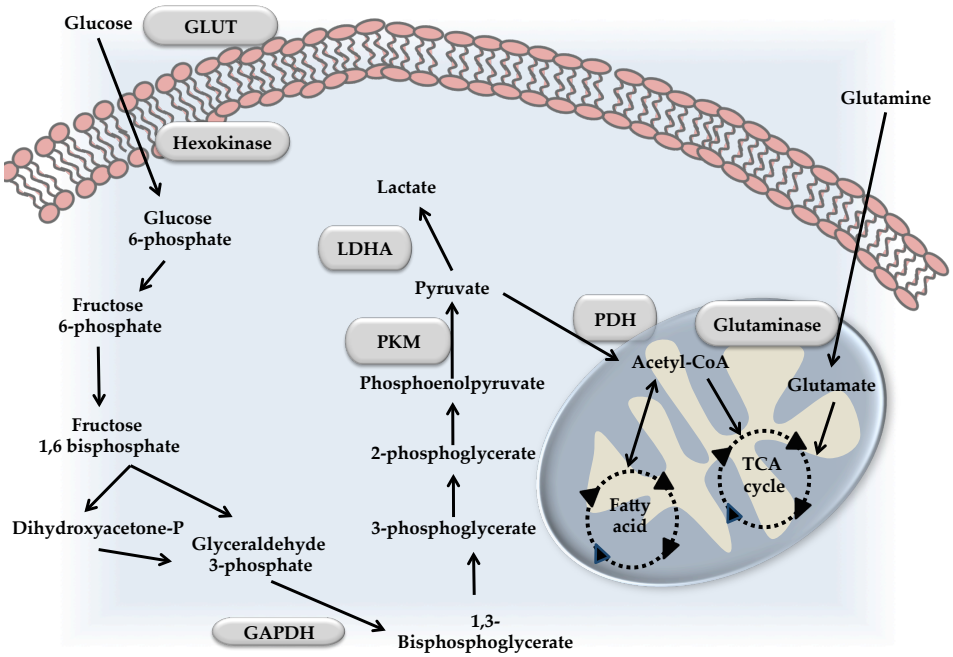

***Figure 2**. Metabolic pathways in cell and mitochondria: Glycolysis, TCA cycle and Fatty acid β-oxidation.*

Glycolysis and fatty acid β-oxidation are catabolic reactions that generate acetyl-CoA. Glucose can be processed through glycolysis for production of ATP and pyruvate, which is then fermented to lactate or enters into the mitochondrion-localized TCA cycle, generating electrons that are transported through the electron transport chain. Glutamine is handled to form glutamate, which is processed to maintain the TCA cycle.

***Abbreviations:** GLUT-Glucose transporter; GAPDH-Glyceraldehyde 3-phosphate dehydrogenase; PKM-Pyruvate kinase; LDHA-Lactate dehydrogenase; PDH-Pyruvate dehydrogenase*

1.2 Mitochondrial biogenesis

Metabolic defects may originate in a decrease of transcription of mitochondrial proteins or in increased mitochondria degradation mediated by mitophagy. Hence, in some specific pathologies, increased mitochondrial biogenesis can be one solution to prevent metabolic disorders [13].

Mitochondria have their own genome, mitochondrial DNA (mtDNA), encoding 13 subunits of the oxidative phosphorylation system (OXPHOS) from which transcripts are produced and then processed to yield individual mRNAs. Two transcription factors play a key role in nuclear–mitochondrial communication: nuclear respiratory factor 1 or 2 (NRF-1, NRF-2) and mitochondrial transcription factor A (TFAM). Both factors lead to transcription of nuclear genes that encode for components of OXPHOS, initiating mtDNA transcription and replication [14,15].

Amongst the several important transcription factors, the transcriptional co-activator of peroxisome proliferator activated receptor gamma (PPARg), known as PGC-1α is of uttermost importance. PGC-1α is described as a co-activator that regulates genes involved in energy metabolism, including fatty acid β-oxidation enzyme expression, but also can activate mitochondrial biogenesis through its interaction with NRFs [16]. PGC-1α gene expression increases during cardiac failure, and overexpression of PGC-1α in cardiac myocytes in culture or in cardiac muscle of transgenic mice leads to increased mitochondrial content over-expression of fatty acid β-oxidation enzymes and respiration [12,17]. PGC-1α thus behaves as a key regulator of mitochondrial function capacity and participates in the transduction of physiological stimuli to energy production in the heart.

1.3 Doxorubicin as a Cardiotoxic Anti-cancer Agent

Doxorubicin (DOX) (Fig. 3) is an antitumor anthracycline antibiotic, isolated from colonies of *Streptomyces peucetius caesius* in 1967 [18,19].

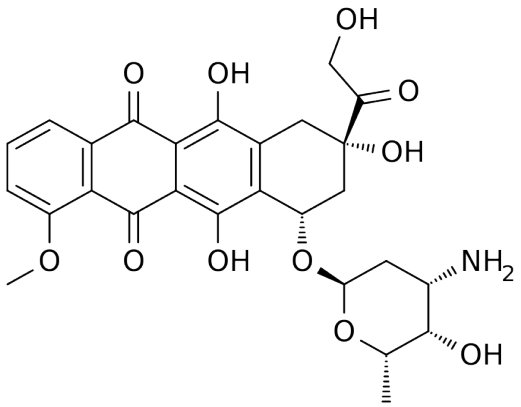

***Figure 3**. Doxorubicin chemical structure, $C_{27}H_{29}NO_{11}$.*

Doxorubicin is one of the most-used antitumor anthracyclines, being highly active against an extensive variety of neoplastic diseases, even at lower doses [19]. The first *in vivo* studies performed with DOX in cancer animal models demonstrated inhibition of neoplastic proliferation and increased animal survival [18]. Doxorubicin efficacy was tested in humans on different types of malignancies, such as leukemias, lymphomas and solid tumors, including gynecological, urogenital, endocrine, stomach cancer, as well as Ewing and Kaposi's sarcomas [19,20]. The mechanisms of action of DOX in tumor cells essentially involve interaction with the nucleus, mitochondria and biological membranes, although some of these targets are also affected in non-target cells as well. The main mechanisms that have been suggested for DOX anticancer effects include: a) DOX intercalation into DNA, leading to inhibition of protein synthesis and DNA replication; b) generation of reactive oxygen species (ROS), leading to DNA damage and/or lipid peroxidation; c) DNA cross-

linking, binding and alkylation; d) interference with DNA unwinding or DNA strand separation and helicase activity; e) direct membrane effect with disruption of the bilayer structure; and f) initiation of DNA damage via inhibition of topoisomerase II [21,22].

During preliminary toxicity tests in patients, several side effects were detected, including nausea, vomiting and fever [19]. Furthermore, a significant incidence of cardiovascular side effects namely hypotension, tachycardia, arrhythmias, and ultimately congestive heart failure were also described [23]. Acute DOX side effects, including pericarditis-myocarditis or arrhythmias, are generally reversible and clinically manageable [24,25]. However, the mortality of patients that develop congestive heart failure after DOX chronic treatment can be as high as 50% [26], increasing significantly when cumulative doses higher than 500mg/m^2 [23].

1.4. Doxorubicin Cardiotoxicity – From Molecular Events to Cardiomyopathy

1.4.1 Doxorubicin-induced cardiac toxicity – from acute to chronic to delayed

Acute DOX injury occurs rapidly after a single dose or course of therapy, with clinical manifestations occurring from a few minutes to a week of treatment [27]. These manifestations may include transient electrophysiological abnormalities, observed in 20% to 30% of patients, which comprise nonspecific ST and T wave changes, T wave flattening, decreased QRS voltage and prolongation of QT interval, sinus tachycardia, and supraventricular arrhythmias [23]. Although rare, pericarditis

or myocarditis syndrome and acute left ventricular failure have been observed in some fatal cases [28-30]. On the other hand, chronic side effects are more serious, irreversible and involve the development of cardiomyopathy and ultimately congestive heart failure [31,32].

The prevalence of left ventricular contractile dysfunction in patients with a cumulative DOX dose of approximately 430 to 600 mg/m^2 is about 50-60%, in which a significant incidence of cardiomyopathic episodes is observed. The incidence of heart failure is nearly 2% with a cumulative dose of 300 mg/m^2 but rapidly increases to 20% at cumulative doses in excess of more than 550 mg/m^2 [33-37].

Early-onset, chronic, progressive cardiotoxicity usually occurs within a year of treatment, persisting or progressing even after discontinuation of anthracycline therapy, leading to chronic dilated cardiomyopathy in adult patients and to restrictive cardiomyopathy in pediatric patients [33,38]. DOX-induced left ventricular dysfunction is generally insidious in onset and progressive in nature with cumulative dosage [35,36]. If treatment with DOX is continued after the onset of subclinical left ventricular dysfunction, a rapid decline in left ventricular function and heart failure occurs [37]. On the other hand, late-onset progressive cardiotoxicity results in ventricular dysfunction, heart failure and arrhythmias years or even decades after chemotherapy occurred, suggesting the need for a continuous follow-up of the cardiac status of patients who received anthracyclines [30,38].

1.4.2 Risk factors

A number of risk factors contributes to DOX-induced cardiotoxicity, including the total cumulative dose, total dose administered during a day or a course of treatment, rate and schedule of administration, mediastinal radiation, age, gender, concurrent administration of cardiotoxic agents, combined therapy, prior anthracycline chemotherapy, history of pre-existing cardiovascular disorders, hypertension, liver disease, and electrolyte imbalances such as hypokalaemia and hypomagnesaemia [30,34,39,40] (Fig. 4).

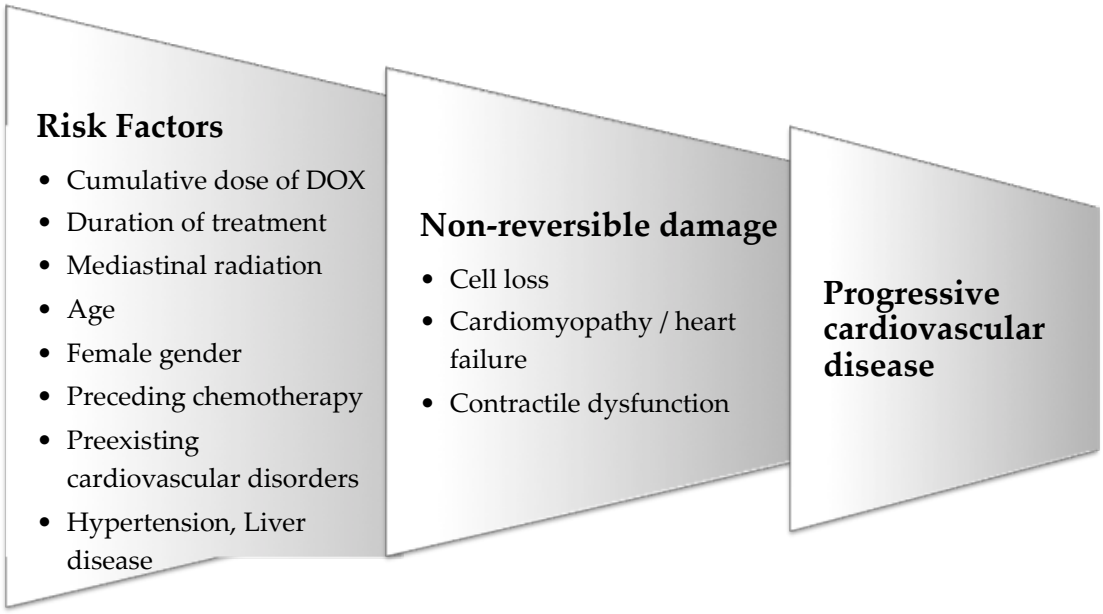

***Figure 4**. Risk factors associated with DOX cancer therapy leading to cardiotoxicity.*

Age seems to be the most significant risk factor since children, adolescents and elderly patients are the most susceptible to the cardiotoxic effects of DOX chemotherapy [41-43]. Particularly in children, the reason may be related to specific effects on pluripotent stem cells in post-natal hearts, disturbing their ability to differentiate to functional cardiomyocytes. Similarly to other undifferentiated cells,

cardiac stem cells can be more sensitive to DOX and their death will limit the regenerative capacity of heart [44]. It is possible that DOX-induced loss of cardiomyocytes together with an early damage to cardiac stem cells in pediatric patients can result in a permanent compromised cardiac function among long-term cancer survivors. Loss of myocytes and impaired cardiac growth results in inadequate left ventricular mass and may result in the development of cardiomyopathy a year or more after cessation of chemotherapy [45]. Nevertheless, cardiomyocyte atrophy and myofiber disarray may also contribute to cardiac dysfunction observed in DOX-treated juvenile patients or animal models [46]. Regarding older age groups, aging impacts DOX pharmacokinetics, which is particularly evident in the heart [47,48]. Age is also highly correlated with drug clearance, which is reduced in the heart tissue of elderly patients and may contribute to DOX-induced cardiotoxicity [49]. This is critically important as post-mortem analysis of patients who were treated with DOX showed high accumulation of this drug in the cardiac tissue vs. other muscle tissues [50]. Age-related DOX cardiotoxicity was also attributed to the decline in regional blood flow with age which may be responsible for the altered DOX concentration-time course in plasma and heart tissue [49].

Taking into account the diverse risk factors, it is important that each patient treated with DOX undergoes an assessment of baseline cardiac function before chemotherapy, a regular monitoring during treatment and a close lifelong follow-up in an attempt to minimize the risk of development of irreversible cardiotoxicity [36,51].

Doxorubicin-induced cardiotoxicity often results in enlarged hearts, with dilation of all chambers and mural thrombi, commonly observed in both ventricles [52]. Also, multifocal areas of irregular and interstitial fibrosis and spotted cardiomyocytes with vacuolization were already detected [52]. Myofibrillar loss and vacuolar degeneration have been two classical ultrastructural features of DOX cardiotoxicity [53]. Despite the large array of clinical outcomes resulting from DOX toxicity, it has been suggested that a specific, progressive, and subclinical anthracycline injury to the human heart cannot be consistently detected by conventional tests [53].

1.4.3 The beginning of the end: Doxorubicin-induced cardiac ultrastructural alterations

At a cellular and molecular levels, DOX cardiotoxicity has been associated with ultrastructural changes in cardiomyocytes, namely cytoplasmic vacuolization involving distention of T-tubules and sarcoplasmic reticulum; myofibrillar disorganization and loss; mitochondrial alterations including swelling [51,54-56] and cristae disruption [57], increased number of lysosomes [58] and clumping of chromatin and nucleoli shrinkage along with segregation of granular and fibrillar components [59]. Doxorubicin disturbs the cellular cytoskeleton and microtubular polymerization, as observed by reduced expression of α-actin, myosin light and heavy chains, tropomyosin, troponin I, troponin C, and desmin [52,59-61]. Doxorubicin treatment also affects adhesion proteins, such as matrix metalloproteinase MMP-2/MMP-9 gene expression [62], resulting in cell detachment [63]. General mitochondrial and fibrilar disorganization was also detected in human

samples [64]. Most of these effects were replicated *in vitro;* DOX toxicity on H9c2 cardiomyoblasts includes mitochondrial depolarization and fragmentation and disorganization of the mitochondrial network, structure and cytoskeleton [59]; however, supraclinical concentrations of DOX affect mitochondria more drastically, which is reflected by mitochondrial swelling and loss of mitochondrial cristae in the same model [59]. Doxorubicin toxicity in mitochondria has also been observed in other tissues including brain [65], lung [66] and liver [67,68], although not as extensively as in the heart.

1.4.4 The beginning of the end: is doxorubicin-induced oxidative stress in cardiac mitochondria the culprit?

From a mechanistically point of view, DOX-induced cardiotoxicity has been attributed to a number of causes, including increased oxidative stress, direct inhibition of key transporters involved in ion homeostasis (such as Na^+-K^+ ATPase and Ca^{2+} ATPase), which results in alterations in cellular calcium homeostasis, increased cytosolic iron accumulation, mitochondrial failure, inhibition of nucleic acid and protein synthesis, release of vasoactive amines, changes in adrenergic function, lysosomal alterations, and apoptosis [51,65,69].

The long list of mechanisms that have been proposed to explain the development of DOX-induced cardiomyopathy demonstrates that the cause is probably multifactorial and complex, but most of these alterations may be attributed to free oxygen radicals production, mitochondrial and bioenergetic failure, which may ultimately develop in apoptosis [51] (Fig. 5).

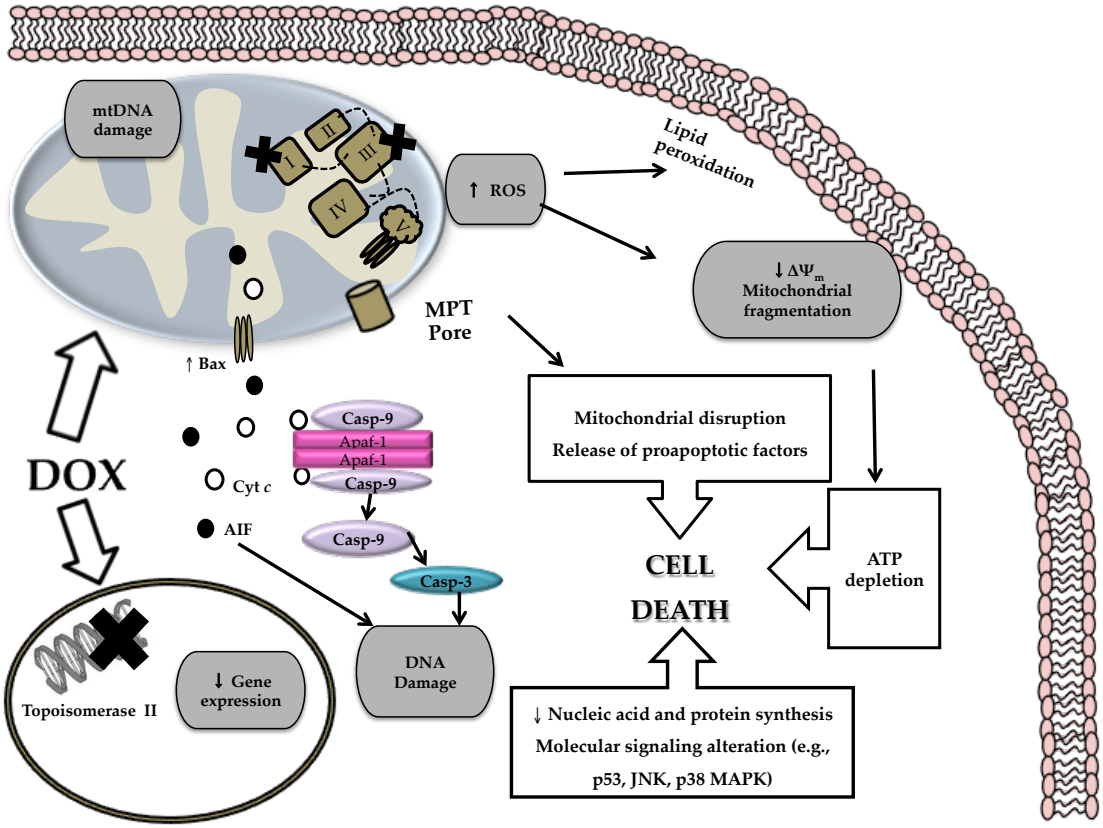

Figure 5. *Most consensual model for DOX cardiotoxicity.*

Data suggests that a large component of DOX-induced cardiotoxicity is mediated by a redox cycle on mitochondrial complex I. Increased ROS generation by DOX redox cycle has several negative consequences, such as mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption, MPT pore formation, ATP depletion and peroxidation of cellular membranes. Marked mitochondrial morphological disturbances induced by DOX include cristae disruption, matrix disorganization and mitochondrial fragmentation. MPT pore-induced outer membrane rupture due to osmotic swelling or permeabilization of the mitochondrial outer membrane mediated by pro-apoptotic proteins including BAX can lead to the release of cyt c and AIF. DOX also interferes with topoisomerase II, inhibiting DNA replication and preventing the repair of damage DNA strands. Finally, persistent down-regulation of gene expression can be another consequence of DOX toxicity. All of these events may lead to cell death.

Abbreviations: *DOX-Doxorubicin; Casp-Caspase; $\Delta\Psi_m$-Mitochondrial transmembrane potential; ROS-Reactive Oxygen Species; MPT-Mitochondrial Permeability Transition; Cyt c-Cytochrome c; AIF-Apoptosis Inducing Factor; JNK-c-Jun N-terminal kinase; MAPK-Mitogen-activated protein kinase; ATP Adenosine triphosphate.*

The heart is particularly susceptible to DOX-induced oxidative damage because of the large density/volume of mitochondria, which are both important sources and targets of ROS (see below), its elevated rate of oxygen consumption, and the lower amount of antioxidant defenses compared with other tissues, such as the liver [57,70,71]. In fact, cardiomyocytes express low levels of catalase [72], while selenium-dependent GSH-peroxidase-1 is readily inactivated after exposure to DOX [72], which also decreases cytosolic Cu-Zn superoxide dismutase [73]. A decrease in the content/activity of antioxidant enzymes may represent a common response to DOX treatment [52], at least in a specific time-point. But what is the relationship between DOX and oxidative stress?

One major hypothesis for DOX toxicity is based on the generation of oxidative stress through interaction with iron and oxygen. With an univalent redox potential of approximately -320 mV, DOX is a favorable substrate for reduction by a number of oxidoreductases within the cell, including NADPH-dependent cytochrome P450 reductase [74], NADH-dehydrogenase of mitochondrial complex I [75,76] and assorted soluble oxidoreductases present in the cytoplasm, including xanthine oxidase [77]. Doxorubicin can be reduced by the eNOS reductase domain, enhancing superoxide formation [78]. Also, it was reported that the K_m for the reduction of DOX to a semiquinone free radical by eNOS is 10- to 100-fold lower than the one reported to cytochrome P450 reductase and NADH dehydrogenase [78]. As the cardiac tissue has a high number of mitochondria per cell (up to 35% of the cell volume) when compared with other tissues, it is considered that the main enzyme involved in DOX redox cycling is complex I (NADH cytochrome *c* oxidoreductase) [35]. Specifically,

DOX is reduced by complex I, forming a highly reactive semi-quinone, initiating a redox cycle after reacting with oxygen and releasing ROS in the process [75] (Fig. 6).

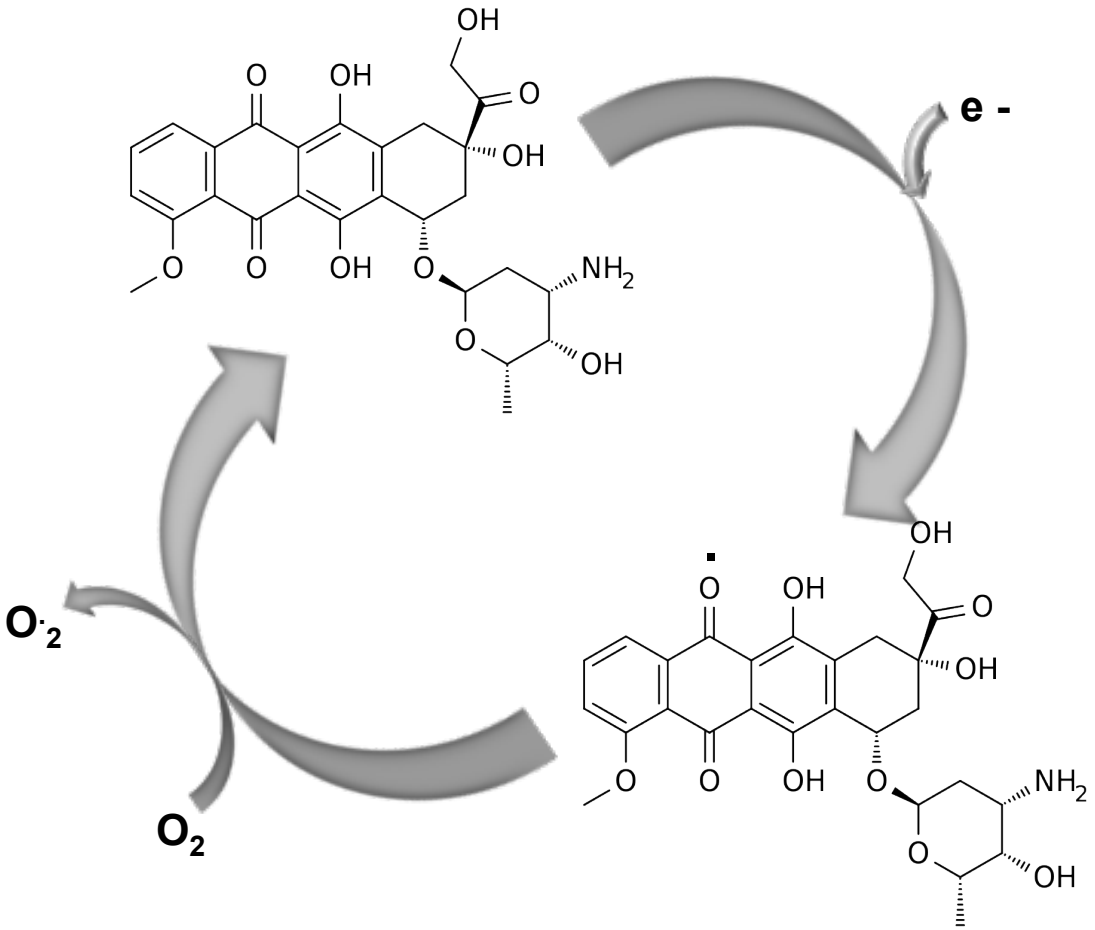

***Figure 6**. Redox cycling of Doxorubicin.*
Univalent reduction (e-) of Doxorubicin to a semiquinone radical. The radical is able to auto-oxidize in the presence of oxygen to generate superoxide anions, reverting to the parent compound.

Doxorubicin also coordinates with free transitional metals, such as iron, to form metal coordination complexes and stimulate production of partially reduced forms of oxygen [79]. Doxorubicin-induced oxygen free radicals can damage phospholipids in biological membranes, increasing the cell membrane permeability and inactivating membrane receptors and other enzymes [80,81]. Doxorubicin also presents a strong affinity for cardiolipin [82,83], one of the most abundant phospholipids in the inner

mitochondrial membrane and which is required for the activity of respiratory chain enzymes such as cytochrome *c* oxidase and NADH cytochrome *c* oxidoreductase [84]. The formation of drug-lipid complexes leads to an inhibition of oxidative phosphorylation because cardiolipin can no longer act as co-factor for mitochondrial enzymes [85]. Binding of DOX to cardiolipin suggests a strong affinity for mitochondrial membranes. It remains to be determined whether this contributes to a buildup of a DOX gradient across mitochondrial membranes. In other words, it is still unclear the percentage of total DOX which crosses the inner mitochondrial membrane and gets reduced by complex I in the matrix side.

Since evidences point out to a preferential interaction of DOX with cardiac mitochondria, it is not surprising that several mitochondrial alterations including stimulation of respiratory state 4 and decrease in respiratory state 3, as well as a decrease in the respiratory control ratio (RCR), have been measured in different models [54,68,86-88]. Doxorubicin-increased ROS generation resulted in the oxidation of lipids, proteins and signaling molecules [89-91]. A decrease in mitochondrial membrane potential has also been described [91].

In several *in vitro* and *in vivo* models, one particular aspect of DOX toxicity is a loss of mitochondrial calcium loading capacity [54,67,68,86,87]. Cardiac mitochondria isolated from DOX-treated rats do not accumulate the same amount of calcium as their control counterparts and it was demonstrated that the loss of calcium loading capacity was due to increased induction of the mitochondrial permeability transition (MPT) pore [67,92], which is a deleterious phenomenon for mitochondria and cells, and which may initiate cell death induction [93]. Oxidative stress, calcium overload or irreversible mitochondrial depolarization result in MPT induction, and which

results in increased permeability of the inner mitochondrial membrane (IMM), leading to the opening of non-selective protein pores, which allows the passage of smaller molecules below 1.5 kDa [6] (Fig. 7).

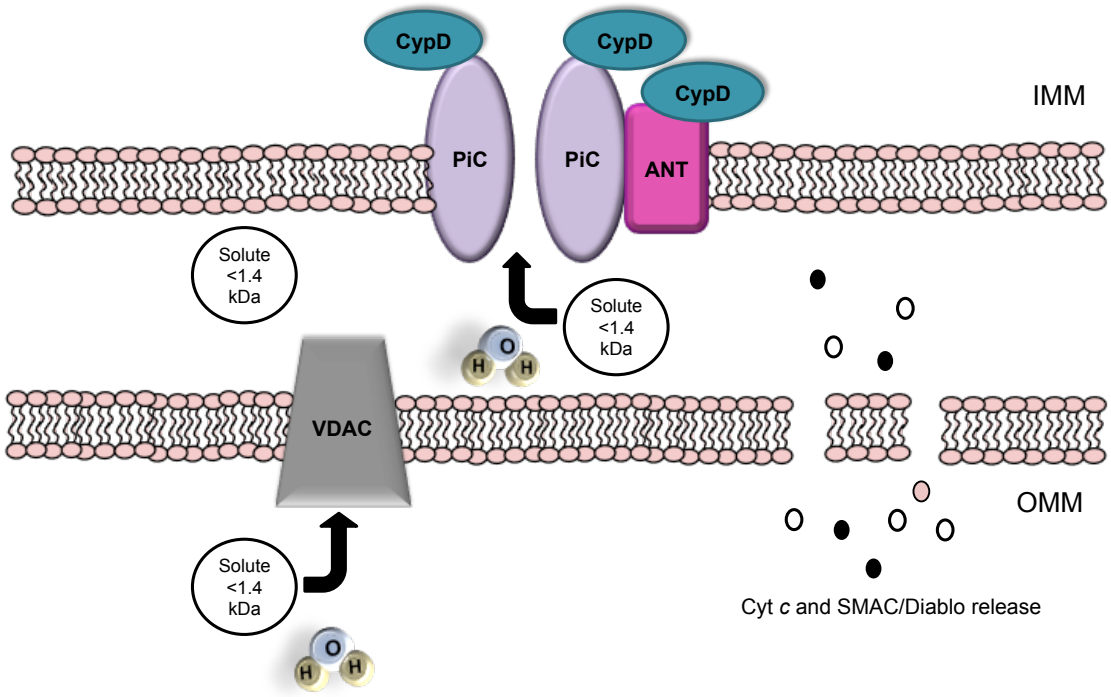

Figure 7. *Mitochondrial Permeability Transition (MPT) pore structure.*
It was recently proposed that the MPT pore is composed/regulated by the matricial protein Cyclophilin D (CypD) and by the mitochondrial phosphate carrier (PiC), with the Adenine Nucleotide Translocator (ANT) probably having a regulatory role. In addition, the MPT pore can be regulated by other components, such as hexokinase, creatine kinase (CK) and peripheral-type benzodiazepine receptor (PBR). Both antiapoptotic and proapoptotic members of the Bcl-2 family modulate the activity of MPT pore (antiapoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-X_L, inhibit pore opening while proapoptotic Bcl-2 family members, such as Bax, Bak and Bid, can induce MPT pore opening). Also, MPT pore opening can be inhibited by CypD ligands, such as Cyclosporin A (CsA). The opening of MPT pore leads to a collapse of transmembrane mitochondrial transmembrane potential ($\Delta\Psi_m$) and favors the release of apoptogenic proteins, such as cytochrome c (Cyt c) and SMAC/DIABLO (Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with low PI).

Mitochondrial permeability transition pore opening leads to mitochondrial osmotic swelling and structural damage to mitochondria. The exact molecular nature of MPT

is still a matter of debate. Although several authors initially supported the idea that the MPT pore was formed by the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and cyclophilin D (Cyp D), it has become clear that Cyp D is the only essential component of the MPT pore [94-96]. Work performed in knockout (KO) animals demonstrated that mitochondria from ANT- and VDAC-KO animals still undergo the MPT [95,97-99]. Recent ideas suggest that the phosphate carrier (PiC) is instead the better candidate for the MTP pore structural component [95,100-102]. Several proteins can regulate the MPT, including creatine kinases (CK), hexokinase, Bcl-2 family proteins and peripheral-type benzodiazepine receptor (PBR) [103]. The opening of MPT pore can trigger the release of proapoptotic proteins such as cytochrome c or SMAC/DIABLO due to rupture of the outer mitochondrial membrane or due to the recruitment of proapoptotic proteins [95].

Increased MPT pore opening in DOX-treated animals can be dependent on the higher basal oxidation of specific thiol residues in mitochondrial proteins [104]. DOX-induced cardiac MPT pore is prevented by the use of several specific inhibitors such as ATP or Cyclosporin A (CsA) [105]. Alterations induced by DOX on the ANT can also lead to alter opening of the MPT pore through an apparent regulatory mode and to decreased oxidative phosphorylation due to its ADP/ATP shuttling activity [86,106,107]. Inhibition of the ANT in its adenine nucleotide translocator function can result in an inhibition of respiratory state 3, which is observed in several *in vitro* models [108]. The fact that loss of calcium loading capacity was observed in so many biological models for DOX-induced toxicity led to the cunning idea that this is in fact an early and sensitive marker for DOX-induced cardiotoxicity [54]. Nevertheless, evidence also exists for a primary mitochondrial membrane depolarization, which

precedes a matrix calcium overload and increased ROS production [91]. Whether the primary event is mitochondrial depolarization or the loss of calcium loading capacity caused by thiol oxidation remains to be confirmed.

Deleterious alterations in cardiac mitochondrial function, including decreased calcium loading capacity and gene expression profile have been demonstrated in rodent models to be irreversible (at least until the studied time point) [92,109,110], which again supports the notion that DOX toxicity in the myocardium is cumulative and irreversible in nature.

Doxorubicin also damages mitochondrial DNA (mtDNA) [111], by forming adducts with that circular genome and therefore disturbing the process of synthesis of mitochondrial machinery [112-114]. The oxidation of mtDNA [115,116] can result in a defective respiratory chain, which will then be unable to respond to high energy demands and produce a higher leakage of electrons to molecular oxygen, increasing ROS production, which then perpetuates the mitochondrial bioenergetic failure.

The above–described strongly suggests that DOX-induced oxidative stress can an important factor in the development of cardiac toxicity. If this is true, than a prophylactic measure to stop cardiac deterioration would involve the use of antioxidants. In vitro data indicates that antioxidants are marginally effective to counteract DOX-induced toxicity on cardiomyoblasts [117]; other data confirm that results with antioxidants yield mixed results [118-121]. One of the first antioxidants tested against DOX cardiotoxicity was vitamin E [71]. A bolus injection of vitamin E before DOX administration prevented the typical cardiac alterations in mice [122]. Other studies showed conflicting results regarding the efficiency of vitamin E and further research showed that vitamin E was cardioprotective only against acute

cardiotoxicity resulting from anthracycline chemotherapy but offered no protection against the development of chronic cardiomyopathy [123]. Berthiaume *et al.* [124] showed that vitamin E–succinate prevented cardiac oxidative stress but not mitochondrial alterations. Interestingly, vitamin-E-succinate [125] and vitamin E [126] increased the anti-cancer effects of DOX in tumor cell lines.

Carvedilol, a clinically approved drug that acts as a nonselective β-adrenergic blocker agent and also blocks α_1-receptor with vasodilatory properties, has also been described to have potent antioxidant activity [68,121]. In different models, carvedilol prevented DOX-induced left ventricular dysfunction [68,121,127,128] and DOX-induced apoptosis of cardiac cells, as well as hydrogen peroxide generation [121] Comparing effects of carvedilol with atenolol, another β-adrenergic blocker agent lacking antioxidant activity, it was concluded that carvedilol protective effects against DOX toxicity resulted predominantly from the antioxidant effect and not from the β-adrenergic blocker activity, since atenolol did not prevent or inhibit DOX-induced ROS production and cardiac apoptosis [121,127,129]. Nevertheless, it may be speculated that the cardiac sparing effect of carvedilol, resulting from β-adrenergic blocking activity, may actually decrease some of the metabolic burden placed on already damaged mitochondria, thus synergizing with the antioxidant effect. Several other studies demonstrated that carvedilol was effective in decreasing cardiac mitochondrial damage induced in a subchronic model for DOX toxicity, including preventing the loss of mitochondrial calcium loading capacity [68,129]. Interestingly, carvedilol was shown to inhibit a hypothetical cardiac NADH dehydrogenase in the outer leaflet of the inner mitochondrial membrane [130], which was proposed to be another possible mediator of DOX redox cycle in the heart [131].

However, further studies are necessary to verify if the protective action of carvedilol may be useful not only in the prevention of DOX-induced cardiotoxicity, but also to confirm that carvedilol does not interfere with the DOX antitumor activity [121,127,132,133].

Despite the positive impact of some antioxidants against DOX cardiotoxicity, the truth is that no one has demonstrated without doubt that macromolecular damage resulting from DOX-generated mitochondrial oxidative stress is the culprit for the cardiomyopathy that results from treatment or if instead mitochondrial oxidative stress only has an important role in the initial acute toxicity observed.

Dexrazoxane is an intracellularly-activated chelating agent with structural similarity to EDTA and was found to have cardioprotective effects [134]. Dexrazoxane prevents functional damage of cardiac mitochondria initiated and perpetuated by ROS, which leads to a reduction of the incidence of DOX-induced cardiomyopathy in humans and animal models [135-137]. Despite the advantages of using dexrazoxane against DOX toxicity, this cannot be seen as the proof that oxidative stress is indeed the main culprit of DOX cardiotoxicity. In fact, equally or more potent iron chelators such as desferrioxamine [138] or deferasirox [139], have failed to afford the same degree of protection. This may imply that iron has no role in oxidative stress-mediated DOX toxicity, as previously suggested [140] or that indeed oxidative stress has a minor role in the entire process of DOX cardiac toxicity. Also, patients treated with dexrazoxane may have a lower tumor response rate [141,142]. In one clinical trial, patients treated with dexrazoxane presented lower response rates (48% vs. 63%) and earlier disease progression compared with patients receiving placebo [143].

Interestingly, a recent paper confirms that global oxidative stress may not be a major execution step in the development of anthracycline-induced cardiomyopathy; instead, it is proposed that anthracyclines disturb mitochondrial biogenesis in response to energy deficits [144]. Although this work has been performed with daunorubicin and not with DOX, we may speculate that a similar result may be obtained with the latter. Still, positive results have been obtained in animal models over-expressing antioxidant enzymes including metallothionein [145], glutathione peroxidase [146] and catalase [147]. Although the disperse data may appear difficult to conciliate, one possibility is DOX toxicity results in localized oxidative stress, which, despite not causing major macromolecular damage, disturbs the redox equilibrium in different cellular compartments during and after DOX treatment. This localized oxidative stress would interfere with compartmentalized cell signaling and with different redox pairs GSH/GSSG and cysteine/cystine [148,149]. Differences in the efficacy of iron-chelators or antioxidants may be explained by their different intracellular localization/accumulation or even by the fact that antioxidants can disturb an otherwise normal redox balance in specific cell compartments [150]. This would result in an apparent lack of protective effect against DOX cardiotoxicity. If this hypothesis is confirmed, the development of novel mitochondrial-directed antioxidants/iron-chelators could help improving DOX phenotype. One example is MitoQ, which prevented inactivation of mitochondrial complex IV by DOX [151].

Despite the promising results of multiple antioxidants, a background problem remains: obvious protection against DOX cardiotoxicity observed in animal models rarely translates into a similar protection in humans. Different drug distribution, metabolism and clearance, dissimilar regulation of redox systems at the genetic level

or even distinct cardiac metabolism may all be the genesis of inter-species differences.

1.4.5 The beginning of the end: does DOX-induced loss of cardiac cells contribute to cardiomyopathy?

Enhanced MPT pore induction resulting from DOX treatment can result in cell death [152,153]. In fact, a rapidly expanding body of evidence supports the concept that both endothelial cell and cardiomyocyte apoptosis and necrosis are involved in DOX-induced toxicity [154,155] The molecular mechanisms underlying DOX-induced cardiomyocyte death comprise the excess generation of ROS [121,152] and other ROS-independent mechanisms, including the generation and accumulation of ceramide [156] or priming of Fas-mediated apoptosis [157].

DNA damage and signaling pathways involving the tumor suppressor p53 and mitochondria can also be involved in DOX-induced cardiac cell death [117,158]. Doxorubicin not only induces DNA damage and oxidative mitochondrial damage, but also induces nuclear translocation of p53 [103]. Mitochondrial dysfunction in DOX-treated H9c2 cardiomyoblasts was concluded to be secondary to p53 activation [117]. Upon activation, p53 induces the expression of genes associated with cell arrest, DNA repair and apoptosis [103]. Additionally, DOX activation of mitochondrial p53 [103] can lead to HIF-1 inhibition, [159] and can promote the decrease of heart mass via p53-dependent inhibition of mTOR signaling [158]. Moreover, DNA lesions induced by ROS or directly by DOX interaction increase phosphorylation of p53, up-regulate its downstream genes such as Bax [117,153] and activate JNK and p38 MAPK pathways to induce apoptosis [160]. Confirming the

involvement of the axis p53-Bax, the p53 inhibitor pifithrin-α decreases Bax expression and effectively inhibits DOX-induced apoptosis in H9c2 cells, neonatal rat cardiomyocytes, and mouse hearts [103,161].

An early event also observed in DOX cardiotoxicity is GATA-4 depletion, which also leads to cardiomyocyte apoptosis [162]. GATA-4 is a transcriptional factor critical in heart development that regulates the apoptotic pathway by activating the antiapoptotic gene Bcl-X_L, thus preserving mitochondrial function and integrity [153]. Doxorubicin also inhibits AKT phosphorylation, increasing active GSK3β, a negative regulator of GATA-4 in the nucleus [163]. Also, dephosphorylation of AKT and Bad can activate caspase-3, inducing inter-nucleosomal DNA damage [164].

Cardiomyocyte apoptosis may be a mechanism by which DOX causes a deterioration of cardiac function, as described in different systems [165]. The evidence supports that DOX causes cell death through multiple mechanisms which apparently have mitochondria as initiators [166] or at least magnifiers of the apoptotic signaling. Can we inhibit apoptosis to decrease DOX toxicity in the heart? As with antioxidants, the data is confusing and often yields a mixed message. For example, over-expression of the anti-apoptotic protein Bcl-xL in neo-natal rat cardiomyocytes decreased apoptosis but failed to prevent down-regulation of gene expression and ROS generation [167]. To complicate things further, DOX-induced cardiac cell death may also occur through caspase-independent pathways [168], which leaves no space for the effective use of caspase inhibitors. Targeting mitochondrial dysfunction to prevent apoptosis may also prove of limited value since apoptotic signaling originating from other organelles was also found to be important in the context of DOX cardiotoxicity. One of those organelles was shown to be the sarcoplasmic

reticulum, leading to activation of caspase 12 [169]. As described above, p53 signaling may be also an important drug target to prevent DOX cardiotoxicity [170]. Again, this may also prove of limited value as ablation of p53 via condition knockout was not enough to prevent DOX-induced fibrosis and cytoskeletal alterations [171]. Results showing that the p53 inhibitor pifithrin-alpha may have multiple actions, including decreased expression of different caspases [172] increases the suspicion that some of the reported protective effects against DOX cardiotoxicity [117,161] may be, in part, p53-independent. Also, the notion that DOX may cause senescence in cardiomyocytes, as opposed to apoptosis [173,174], adds another layer of complexity. This new view means that DOX may trigger aging mechanisms in the heart which may, or not, lead to the triggering of apoptotic or even autophagic [175,176] signaling mechanisms. Another problem is that avoiding apoptosis in the cardiac tissue may also result in exactly the same effect in a cancer cell, which was obviously the first target for DOX. Interestingly, pifithrin-alpha increased cell death in neoplastic mouse JB6 cells by increasing p53-mediated apoptosis [177]. This suggests that this compound, regardless of its real selectivity against p53, may contribute to reduce DOX-induced cardiac damage, and at the same time induce death of some tumor types.

1.4.6 Progression of evil: Doxorubicin-induced metabolic remodeling in the heart

The progressive degeneration of mitochondrial bioenergetics during DOX treatment may induce cardiac bioenergetic disruption, which may result in cardiac depression.

In fact, the ATP or phosphocreatine content of hearts from rats chronically treated with DOX is lower, implying decreased bioenergetic capacity [55]. Metabolic readjustments are thus necessary to support myocyte vital functions.

Effects on the global cardiac energetic network or on its individual components have been studied in different *in vitro* and *in vivo* models for DOX-induced cardiotoxicity [108,178]. The impact of DOX on global cardiac metabolism is demonstrated by the fact that DOX not only diminishes overall mitochondrial ATP production, but leads to a rather specific early impairment of CK isoenzymes including mitochondrial CK isoenzymes (MtCK) and AMPK [179,180]. Compromised MtCK leads to impairment of energy channeling and signaling between mitochondria and the cytosol and interferes with mitochondrial respiration [179,180]. Inhibition of AMPK, a highly susceptible target of DOX-induced damage in the heart, blocks mitochondrial import and inhibits the β-oxidation of fatty acids [179]. Also, impaired AMPK signaling reduces the capacity of the cell to initiate a compensatory increase in glycolytic rate and to stimulate glucose uptake [179,181]. Glycolytic enzymes have been shown to be associated with sarcolemmal and sarcoplasmic reticular membranes and functionally coupled to ion transport pathways, including calcium channels [182] or the sodium/proton exchanger [183]. Alterations in glycolytic fluxes may interfere with ion transport and progressively lead to depression of cardiac excitability.

Also, impairment of carnitine palmitoyl transferase I (CPT I) and/or depletion of its substrate L-carnitine was observed during DOX cardiotoxicity [184,185]. Since long-chain fatty acid β-oxidation is inhibited by DOX [186], an increase of total cholesterol, triglycerides and LDL cholesterol in the serum can be a consequence, described already in animal models [50,88]. Both glucose utilization and β-oxidation of long-

chain fatty acids are impaired in DOX-induced cardiomyopathy [52,186,187], which taken to the extreme may imply a general breakdown of energy metabolism after DOX treatment.

Interestingly, a link between general oxidative stress and metabolic responses was already proposed. Following one hour of DOX treatment, lipid peroxidation increased and caused an adaptive response resulting in an increased glucose uptake which is manifested by an increase in GLUT1 protein, presumably to restore cellular energy, establishing a relationship between DOX-induced lipid peroxidation and acute alterations in glucose transport [187]. Early responses such as the induction of glycolytic and TCA cycle genes [188] may be an adaptation response to a general bioenergetic breakdown, although a metabolic switch from oxidative phosphorylation to glycolysis can only provide a short-term solution (and, as described above, the increase in glycolytic fluxes is limited due to DOX off-target toxicity). An increase of lactate production, a sub-product of glycolysis and a likely signal of decreased mitochondrial function and up regulated glycolysis was previously described after DOX treatment [186]. Through nuclear magnetic resonance, analysis of intracellular metabolites, myocardial levels of acetate and succinate were increased in DOX-treated animals as compared to controls, which was correlated with non-enzymatic conversion of pyruvate to acetate and of α-ketoglutarate to succinate, as mediated by DOX-induced free radicals [189]. In fact, accumulation of acetate and succinate were proposed as novel biomarkers for DOX cardiotoxicity [189]. Also, myocardial levels of branched amino acids (BCAAs) valine, leucine and isoleucine are decreased, meaning that BCAAs can be used as an

energy reservoir, resulting from the fact that fatty acid β-oxidation is inhibited and TCA cycle is impaired [189] (Fig. 8).

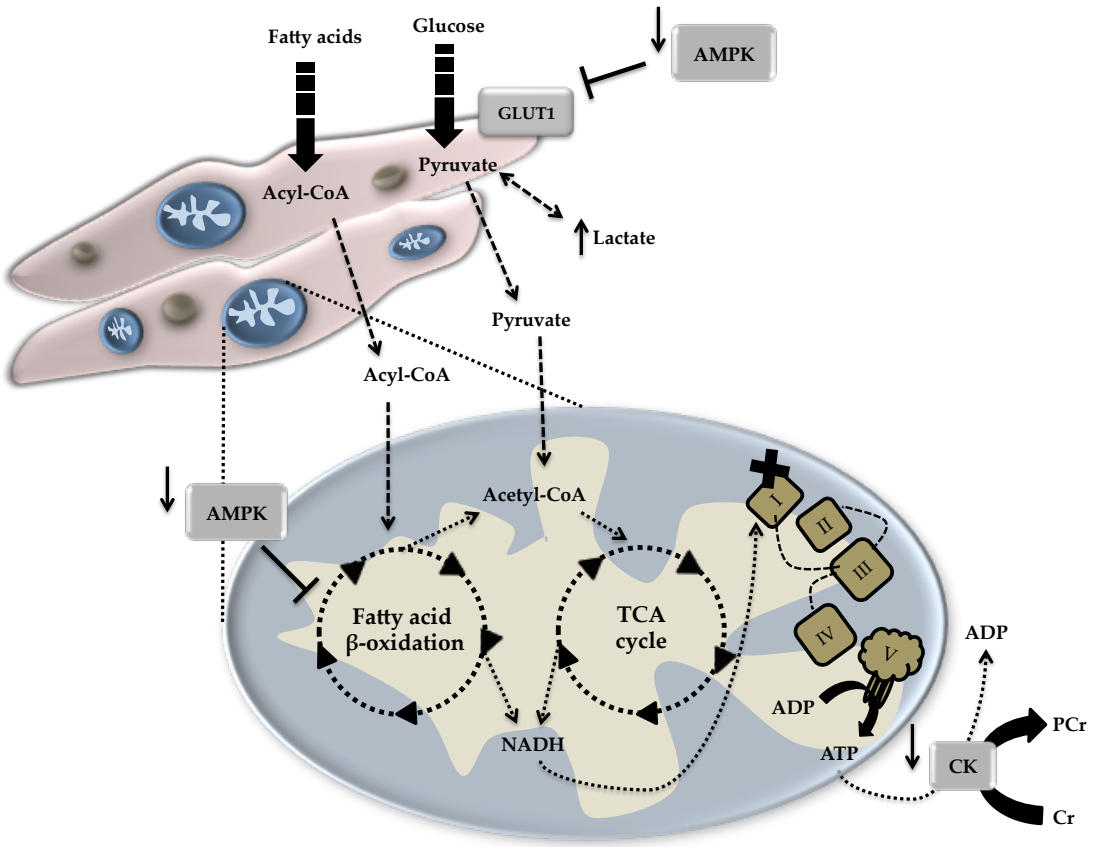

Figure 8. *Metabolic alterations described in DOX-induced cardiotoxicity.*
Cardiac metabolism is altered in different levels by DOX. Besides the disruption of mitochondrial oxidative phosphorylation (OXPHOS), which include inhibition of Complex I activity, also a number of other proteins involved in other metabolic pathways is affected. Mitochondrial CK isoenzymes (MtCK) are mandatory in the channeling of mitochondrial ATP to creatine-phosphate, used as the source of energy in cardiomyocytes, AMPK activity is also inhibited by DOX, which leads to a decline of fatty acid β-oxidation. AMPK down-regulation affects glucose transporter negatively, blocking the compensatory pathway that stimulates glucose uptake following disrupted OXPHOS.
Abbreviations: *DOX-Doxorubicin; AMPK-Adenosine monophosphate-activated protein kinase; GLUT1-Glucose transporter 1; NADH-Nicotinamide adenine dinucleotide; CK-Creatine kinase; PCr-Phosphocreatine; Cr-Creatine; ATP-Adenosine triphosphate; ADP-Adenosine diphosphate.*

The switch from long-chain fatty acid oxidation to alternative substrates is a common feature of several stressful conditions, in which DOX cardiotoxicity is naturally

included and may be an important contributor to the metabolic phenotype observed after DOX treatment, resulting in persistent bioenergetic failure. One logical question is to know the extension of the irreversibility of the metabolic injury/remodeling process and how that affects decreased cardiac resistance to future deleterious stresses. However, very few data are available from studies performed in humans, which is clearly a limitation. An important advancement in this field would be a follow-up of global cardiac metabolism in former DOX patients as compared with untreated subjects, not only during resting conditions but also when subjected to cardiac stress.

Studies involving maintenance of regular cardiac metabolism during DOX therapy by using pharmacological strategies are also scarce with some exceptions. L-carnitine, which has an important role in the import of long-chain fatty acids [190], has been tested aimed at normalizing metabolism during DOX-induced cardiotoxicity. Results showed some success in decreasing DOX cardiotoxicity in several experimental animal models [191,192]. Interestingly, L-carnitine supplementation reversed the decreased expression of the organic cation/carnitine transporter in the cardiac muscle [193] but did not normalize the activities of cardiac CPT-I [194]. Despite this, hyperlipidemia caused by DOX in rats was decreased by L-carnitine [50]. The results appear to suggest that L-carnitine may be operating in multiple levels and organs and that restoring long-chain fatty acid oxidation may not be the primary mechanism by which L-carnitine operates. In fact, the anti-apoptotic role of L-carnitine in a *in vitro* system of DOX toxicity was proposed to originate from direct inhibition of a novel acid sphingomyelinase, which would lead to the generation of ceramide and apoptosis [156]. This particular aspect may be confusing

since it is known that inhibition of mitochondrial fatty acid beta-oxidation at various levels may increase the generation of toxic lipid metabolites, including ceramide, in the cardiomyocyte cytoplasm, leading to cell death [195].

Dexrazoxane reverts the metabolic consequences of DOX treatment in the heart, such as inhibition of fatty acid oxidation and increased glycolytic rate. In this particular case, a defect in the beta-oxidation mechanism itself and not on fatty acid transport was found after DOX treatment [186].

Despite the lack of information from humans and from the degree of the reversibility of metabolic alterations, it may be possible that metabolic depression, associated with sub-clinical mitochondrial limitations, may contribute to decrease the susceptibility of the DOX-treated heart to different types of stresses.

1.4.7 "Second-hit" stress in the Doxorubicin-treated hearts

Since a significant amount of DOX patients are pediatric, one important concern is whether the treated myocardium will have the same behavior when subjected to different types of stress later in life. Evidence from *in vitro* models, e.g. isolated heart mitochondria from DOX-treated rats, suggests that heart mitochondria from this experimental group are more fragile in the presence of calcium and unable to increase respiration facing a demand of ATP, mimicked *in vitro* by the addition of ADP [104]. This suggests that DOX toxicity may remain silent and thus undetectable under unstressed situations, but may become visible and a matter of concern when the myocardium is forced to perform stressful work. In fact, the literature is scarce

when exploring this subject, i.e. there is not a lot of research available on how DOX primes the myocardium to respond differently to different types of stress.

Pregnancy has been considered a potential risk condition for patients that have been previously treated with DOX for childhood cancers. The literature available appears to suggest that pregnancies may have a favorable, complication-free outcome if basal ventricular function is normal before pregnancy. On the other hand, there are risks for the pregnancy if left ventricular function during rest is already deteriorated [196]. Several peripartum cardiomyopathy and heart failure clinical cases have been described so far [197,198], which again shows that patients that were previously treated with DOX in an early age should be followed very closely in the event of a pregnancy. Also, one important point of concern is the fact that offspring from DOX childhood patients may present birth defects or higher cancer incidence. Nevertheless, one available study performed in 405 former childhood cancer survivors, showed no increased rate for any of those end-points [199].

Despite the fact that exercise can prove very beneficial against DOX-induced cardiotoxicity if performed before the treatment [200] (see below), it can also be a source of metabolic overload for an already stressed cardiac muscle. One interesting and recent paper demonstrated that voluntary exercise in young rats, initiated when DOX treatment was started, was able to prevent the persistence of DOX chronic toxicity [201]. Data from patients suggests that at least during the initial three decades of life, exercise is not detrimental to the cardiac function in childhood cancer survivors. Black *et al.* [202] demonstrated that in most of 56 patients aged 9-28 years, 44 patients of which had been treated with 15-483 mg/m^2 DOX, no signs of cardiac alterations were observed during a physical activity protocol. Despite these

promising studies, there are voids in the actual knowledge, one being the exercise capacity of older survivors of DOX-treated childhood cancer. The other important aspect, is to question whether the bioenergetic deficit and accompanying metabolic remodeling in DOX-treated patients is irreversible and whether these bioenergetic deficits can impact the resistance of the myocardium to "second-hit" stresses, not only including exercise and pregnancy, but also the cardiovascular burden posed by obesity, diabetes or a lifestyle of increased emotional stress. These topics are still mostly open for investigation.

1.4.8 Delayed Doxorubicin toxicity: can we stop something under the surface?

As widely described in the previous sections, one particular aspect of DOX toxicity is a delayed component, surfacing years or even decades after treatment [203]. Despite all the knowledge gathered along the last decades, we are still far from identifying the mechanism(s) behind the delayed cardiac toxicity. It is doubtful that traces of DOX may remain in the cardiac tissue years after cessation of the treatment, despite the fact that DOX may bind to mitochondrial cardiolipin [82,83]. Proposed mechanisms to explain delayed DOX cardiotoxicity range from progressive impairment of sarcoplasmic reticulum calcium-handling mechanisms, which was observed 13 and 18 weeks after the termination of treatment [204] to free radical-derived DNA lesions [205]. One should be aware that DOX-induced oxidative lesions on mtDNA [115,116] could also contribute to a progressive decay of mitochondrial capacity, which will cease to be sub-clinical after a certain threshold is crossed.

Another possible explanation may involve the selective removal of progenitor cells in young patients [44], which will later limit the already restricted regenerative capacity of the heart.

Different therapies have been proposed facing data from animal models. These range from antioxidants such as coenzyme Q10 [206], calcium antagonists such as flunarizine [207] or metabolic/antioxidant agents such as trimetazidine [208]. But again, data in humans is still scarce regarding approaches to prevent DOX delayed toxicity. Also, would it be correct to treat a former cancer patient that received DOX with a myriad of protective compounds, not knowing the impact of this supposedly "protective" therapy in normal cardiac physiology? The fact that DOX cardiotoxicity may remain silent for decades does not motivate the cardiologist or the general practitioner into prescribing drugs for something that may not exist. One possible solution may be physical activity, which has been described to decrease DOX toxicity. The next section will provide positive arguments for the use of regular exercise in improving metabolism and especially mitochondrial capacity in the hearts of former DOX patients. Data exists showing the exercise is effective in counteracting acute and sub-chronic DOX cardiotoxicity. If demonstrated, a protective role against delayed DOX toxicity will not come as a surprise.

1.5 Boosting cardiac mitochondrial capacity and defenses with physical activity: limiting the damage

The beneficial effects of exercise in the cardiac muscle are widely reported, and include upregulation of heart antioxidant systems [209], improvement of

mitochondrial function, reduction of the formation of lipid peroxidation by-products [210] and induction of heat shock proteins (Hsp) overexpression [211]. Without surprise, exercise affords cross-tolerance against a series of cardiac pathologies and stresses, including diabetes [212]. It is important to note that exercise can positively modulate some important cardiac defense systems to antagonize the toxic effects caused by DOX treatment [200]. Exercise can present different types of schedules and intensities, including acute treadmill running, short or long term forced endurance training in the form of treadmill running or swimming as well as voluntary physical activity, resulting in different physical responses and mechanical stresses [200,213].

In fact, a single endurance exercise bout can preserve cardiac function and protect the heart against cardiac dysfunction, oxidative stress and lipid peroxidation [214-218]. Wonder *et al.* [214] reported that an acute exercise bout performed 24 hours before DOX treatment protected against cardiac dysfunction. Also, exercise-induced cardioprotection could partly be explained by a reduction in the generation of ROS; however, the mitochondrial mechanisms responsible for this cardioprotection against DOX effects remained vague [214]. Ascensão *et al.* [67] determined the effects of an acute exercise bout on heart mitochondrial function before DOX administration. Exercise prevented most of the deleterious mitochondrial alterations caused by DOX treatment, including the decrease in heart mitochondrial function and increased calcium-induced MPT, besides increasing cardiac mitochondrial SOD activity and preventing apoptotic signaling. Clearly, this study supports that acute exercise protects against cardiac mitochondrial dysfunction. However, due to the high rates of oxygen consumption and ROS production in acute exercise and to the low cardiac muscle ability to neutralize ROS, acute exercise should be recommended

only under controlled circumstances, especially for patients with a previous history of heart disease [219,220].

Endurance training can be defined as the repeated act of exercising to increase aerobic fitness. Long-term endurance training induces many physiological adaptations, which have been described to counteract several pathologies [221,222]. Regular exercise is known to up-regulate cardiac antioxidant systems and to support greater tolerance in the myocardium, with a resulting broad enhancement in its function, both at rest and when subjected to oxidative stress stimuli [220]. Ascensão *et al.* [57,213] demonstrated that endurance exercise, prior to DOX treatment, protected the hearts of rats and mice against DOX cardiotoxicity. Also, training prevented the induction of the MPT pore by decreasing the sensitivity to calcium observed in non-trained DOX-treated rats [57,213]. Moreover, training inhibited a DOX-induced increase in mitochondrial protein carbonyl groups and lipid peroxidation [223], and Bax, Bax/Bcl-2 ratio, induction to mitochondrial permeability transition pore and tissue caspase-3 activity [57]. Combined with a protective phenotype, endurance training increased GSH, mitochondrial heat shock protein Hsp-60, whole tissue Hsp-70 [223], and mitochondrial and cytosolic forms of SOD [57,213]. All of these effects resulting from endurance training were able to protect and improve heart mitochondrial respiratory function from the toxic effects of DOX, probably by improving mitochondrial and cell defense systems and reducing cell oxidative stress. All of these modifications at the cellular and ultrastructural levels are reflected in an improvement of cardiac functional parameters. For instance, endurance training prevented DOX-induced decrease in heart rate, left ventricular developed pressure (LVDP) and dP/dtmax [223].

Regrettably, there is a shortage of basic scientific works demonstrating the impact of the diverse forms of exercise in cardiac metabolism, including fatty acid beta-oxidation and glycolytic rates. The promising results obtained with exercise in the context of DOX cardiotoxicity would warrant such an approach. Nevertheless, a word of caution must be said. One size does not fit all. Patients should be carefully seen by a cardiologist before starting an endurance training protocol and during several time-points. Our limited knowledge of the mechanisms that exercise activates to afford cell protection, combined with the unknown involving DOX delayed toxicity, inhibits the use of physical activity to prevent the latter before further studies.

Interestingly, caloric restriction may resemble the positive effects of exercise. Chen *et al.* [224] demonstrated that caloric restriction induced by the non-metabolized mimetic 2-deoxyglucose (2-DG) antagonized DOX-induced rat neonatal cardiomyocyte death. 2-deoxyglucose decreased intracellular ATP levels by about 20% but prevented the large deficit of ATP following DOX treatment. Moderate caloric restriction was also demonstrated to be effective by rendering the heart more resistant to DOX toxicity in a rodent model [225]. The effect was not dependent on differential accumulation of DOX in the heart but on multiple pathways, including activation of JAK/STAT signaling, decreased oxidative stress or higher ATP/ADP ratio. Fatty acid oxidation in the myocardium was also increased in this model of moderately restricted diet, which may present a positive impact as well.

1.5.1 Limiting problem in animal studies

Doxorubicin cardiotoxicity remains a huge challenge in oncology. Since DOX causes a progressive deterioration of mitochondrial metabolism, this can serve as a basis not only for the phenotype observed in DOX-treated animal models and humans but also for the metabolic remodeling that results from DOX treatment and whose consequences for long term DOX therapy are still largely unknown. The multi-factorial nature of DOX effects and the domino effect that each molecular event may trigger (Fig. 5), can explain why protective agents that otherwise are promising on paper, fail to provide the expected protection. It is also important to note that some of the observed cardiovascular effects may also be consequence of DOX toxicity on other organs, including the kidneys [226], which although milder than in the heart and affecting a lower number of patients, can further complicate the picture and the efficacy of many cyto-protectants.

Also, basic research should be focused on how cell and mitochondrial metabolism are maintained in an altered state and what are the consequences of that for a later life cardiac failure or decreased resistance to "second-hit" stressful events. It also appears logical that mitochondria appear in the first line for protection by pharmacological and non-pharmacological approaches in order to further prevent metabolic remodeling that may prove deleterious in the long term.

One critical problem with several of the animal studies aimed at understanding the mechanisms behind DOX toxicity is the choice of healthy animals in the vast majority of studies. Most studies, including our own, investigate the cardiotoxicity of DOX in animal models devoid of tumors. This extra physiological burden may alter the

cardiac effects of DOX, with the advantage of studying the effects of different compounds on cardiac tissue and in modulation of DOX anticancer efficacy. In a model bearing more resemblance to the clinical reality, Todorova *et al.* [227,228] observed that oral glutamine administration was able to reduce oxidative damage in the heart of tumor-bearing rats. This study is important for two main reasons. One is the evidence that oral glutamine is able to decrease oxidative damage caused by DOX in the heart but not in the tumor. The second important reason has, of course, to do with the model itself. The authors chose to use an animal model that harbors a tumor. Despite this step forward in ameliorating a biological model, the truth still is that a rat (or mouse) is different from a human, which justifies that protective agents that work in animal models fail to do so in humans [229].

In conclusion, much is yet to be done and further research will continuously be conducted in an attempt to validate the essential mechanisms responsible for DOX toxicity and metabolic/mitochondrial alterations involved therein and to develop new therapeutic strategies to prevent premature cardiomyocyte death in cancer patients that benefit from anthracycline treatment. This is an even bigger challenge for childhood cancer survivors.

Chapter 2: Hypothesis and Aim

2. Objectives

Doxorubicin-induced cardiomyopathy is characterized by changes in both morphology and function of heart mitochondria, including interference with mitochondrial calcium homeostasis at subclinical cumulative doses [35]. In fact, there is growing evidence that correlates the onset and severity of DOX cardiotoxicity with disturbance in heart mitochondrial function and bioenergetics [92,166].

The goal of the present thesis is to further clarify mitochondrial alterations and heart hemodynamic behavior and confirm our hypothesis that mitochondrial dysfunction is the early marker for DOX toxicity by pinpointing defects in the energy machinery of the hearts of DOX-treated rats. The rational of the present study is to use the intact perfused heart model to evaluate the contribution of mitochondria to cardiac performance in both SAL and DOX-treated rats, by using an acute and a sub-chronic DOX treatment regimen.

Our assumption for this strategy is that a lower susceptibility to a determined inhibitor during perfusion with distinct substrates, would represent a more robust (i.e. more capacity) of the targeted pathway(s).

The results obtained with this project will allow a better understanding of the role of mitochondrial dysfunction on the mechanisms by which DOX shows a dose-dependent and cumulative toxicity and allow the development of new strategies to reduce such toxicity.

Chapter 3: Material and Methods

3.1 Chemicals

Doxorubicin hydrochloride ($C_{27}H_{29}NO_{11}$.HCL), chemical purity ≥ 95%(HPLC) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) and prepared in a sterile saline solution, 0.9% NaCl (pH 5.0).

All other chemicals used in this work were of the highest analytical grade and were obtained from Sigma Aldrich Co (Madrid, Spain), unless specified in each section.

Aqueous solutions and buffers were prepared with Milli-Q water (Milli-Q Biocel A10, Millipore, Billerica, MA, USA). Buffers' pH was determined using a pH meter, which was regularly standardized with standard buffer solutions at pH 7.0 and 4.0.

3.2 Animal care

Animal handling were performed in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) and the European Requirement for Vertebrate Animal Research and approved by the Animal Research Ethics Committee at the Center for Neuroscience and Cell Biology.

Male Wistar rats, Crl:Wl (Han) with fifteen weeks of age (acute protocol) or seven weeks of age (sub-chronic protocol) were purchased from Charles River Laboratories (France) and acclimated for one week prior the initiation of experiments, in our accredited animal colony (Laboratory Research Center, Faculty of Medicine, University of Coimbra, Portugal) grouped in type III-H cages (Tecniplast, Italy). Specific environmental requirements were maintained: 22ºC, 45–65% humidity, 15–20 changes/h ventilation, 12 h artificial light/dark cycle, noise level <55 dB. Rats had free access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and

water (acidified at pH 2.6 with HCl to avoid bacterial contamination). The author of this PhD thesis is credited by Federation of European Laboratory Animal Science Associations (category B) for animal experimentation.

3.3 Experimental design

Performing the acute protocol, Wistar rats with 16 weeks age were selected randomly and assigned them in two groups: DOX and SAL treatment. One group received a single dose of DOX, 20 mg/kg by intraperitoneal (i.p.) injection, while the other group received the equivalent volume of the vehicle solution of NaCl 0.9% by i.p. injection. Both treatments were carried out 24h before sacrifice.

For the sub-chronic protocol, 8 weeks old Wistar rats were randomly paired and injected subcutaneously weekly (s.c.) in the scruff or flank with 2 mg/kg DOX or with equivalent volume of vehicle solution of NaCl 0.9% by s.c. injection, during seven weeks. Following the last week of injection, animals were allowed to rest for one week before the sacrifice (Fig. 9).

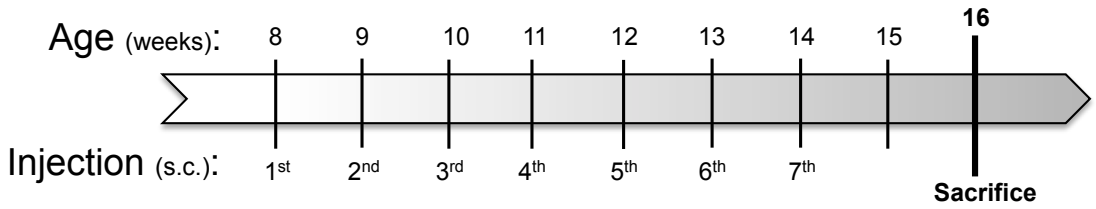

***Figure 9**. Timeline for the sub-chronic protocol DOX treatment on Wistar rats.*

For both protocols, animals were injected during the light phase between 9 a.m. and 11 a.m. and observed daily and weighted at the beginning and at the end of the treatment procedure, for sub-chronic protocol animals were also weighted weekly at

the time of injection.

Animals were euthanized by cervical dislocation followed by decapitation, confirming death of the animal. Extraction of the heart with the aortic stump from the thorax was quickly performed, and heart was washed in a cold (4ºC) Tyrode´s buffer in order to temporarily stop its beating and preserve it from ischemic injury prior to perfusion. The heart was then carefully dried and weighted and used in the perfusion protocol.

3.4 Heart perfusion by Langendorff system

Several techniques were developed over the years to acquire a better knowledge of heart functionality and the development of the isolated perfused heart technique is one of such examples. Starting in 1846, Carl Ludwig and colleagues promoted the study of function of perfused hearts, starting with isolated frog hearts and soon used with cat, dog, rabbit and rat heart [230]. In 1897, Oscar Langendorff created an implanted isolated heart perfusion technique, used for studies on coronary regulation, analysis of toxicological chemical effects or evaluation of cardiac metabolism; a system that still today hears his name [231]. This *ex vivo* technique provides a high reproducibility of results at a relatively low cost [232]. However, during heart perfusion, several parameters must be carefully addressed. Hearts are usually removed from the animal body and cannulated through the aorta to a reservoir outflow that contains an oxygenated perfusion solution (95% O_2 plus 5% of CO_2) at 37ºC. The perfusate, normally a bicarbonate salt solution, is delivered directly in the aorta, leading to a retrograde perfusion direction through the heart at a constant flow, allowing the heart to perform a continuous beat. All the perfusion

apparatus is under a constant temperature, including the heart chamber. Meanwhile, to collect data from physiological, morphological or even pharmacological parameters, a small balloon is usually attached to a pressure transducer is placed in the left ventricle and inflated to allow pressure to be constant inside the heart. Parameters such as heart rate, coronary vascular tone, and contractile function can be analyzed during heart perfusion.

This technique is used to study several heart conditions, such as ischemia, arrhythmias and other heart diseases, heart failure potential activities of drugs or even the effectiveness of heart preservation solutions used for transplantation [233,234].

A Langendorff perfusion system was used with a modified medium, Tyrode´s buffer, that contained (in mM) 140 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 5.0 HEPES and 2.0 $CaCl_2$ with pH adjusted to 7.4 and pre-gassed with 95% oxygen plus 5% of CO_2. To the initial Tyrode´s buffer, we added separately three different metabolic fuels, namely: 10 mM of glucose; 5.0 mM of galactose plus 2.0 mM of glutamine and 1.2 mM of octanoate plus 4.95 mM of malate.

The use of different fuels allowed us to manipulate the metabolic pathways used by the cell. The heart can use different energetic substrates (fatty acids, glucose, lactate, ketones, amino acids). However, mitochondrial ATP is primarily produced by the oxidation of pyruvate, derived from glycolysis and fatty acids. By using a glucose-based buffer, the objective was to stimulate glycolysis [235], the initial stage of glucose metabolism taking place in the cytosol. In this case, theoretically, for each molecule of glucose two molecules of ATP and two molecules of pyruvate are

produced [236], being converted in acetyl-CoA entering the TCA cycle in mitochondria [237].

The main pathway for galactose metabolism is the Leloir pathway [238,239]. In this set of reactions, galactose is transported to the cytoplasm where is converted to galactose-1-phosphate, through galactokinases [240], with subsequent enzymatic reactions converting galactose-1-phosphate to glucose-1-phosphate, which through the activity of phosphoglucomutase is converted to glucose-6-phosphate, following the glycolytic pathway [241]. However, in the absence of glucose, cells are forced to generate ATP through OXPHOS, mostly by glutamine metabolism [242]. Glutamine is an essential amino acid that is converted to glutamate by glutaminase [243]. Glutamate is then metabolized to α- ketoglutarate, a substrate/product of the in TCA cycle, forcing energy production through OXPHOS [244].

Fatty acid β-oxidation is also a source of ATP in the cell. In a normal heart, over 60–90% of ATP produced is derived from the β-oxidation of fatty acids [245]. In the present work the use of octanoate as a fatty acid allowed us to fuel the fatty acid β-oxidation pathway. In this process, octanoate molecules are broken down in mitochondria to generate acetyl-coA that enters in TCA cycle, with malate as an intermediate [245], that allows replenishing of TCA cycle intermediates pools.

After the addition of the different metabolic fuels to the Tyrode's buffer, and for each type of buffer used, three inhibitors were added separately during cardiac perfusion in cumulative doses: iodoacetate; rotenone and potassium cyanide (KCN). These inhibitors were chosen taking into account the interactions with mitochondria and metabolic pathways. Iodoacetate is an inhibitor of glycolysis, by inhibiting irreversibly the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) [246]. Rotenone and KCN, are classics mitochondrial inhibitors, with the first inhibiting complex I and the latter, complex IV [247,248] (Fig. 10).

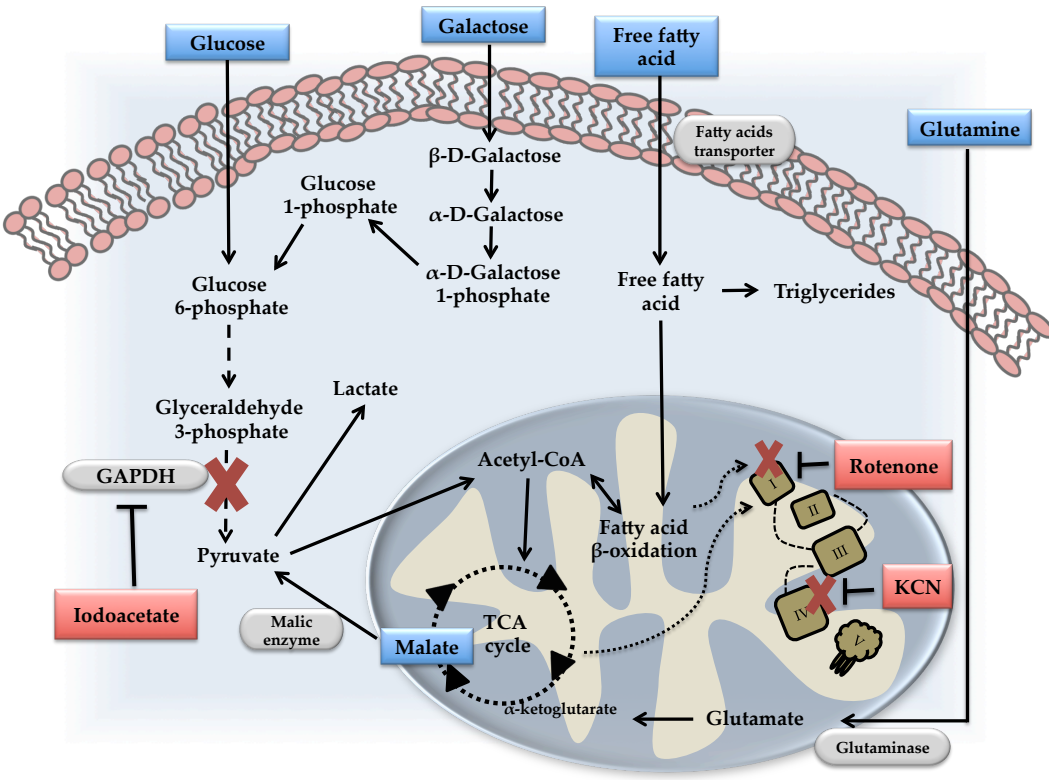

***Figure 10**. Schematic drawing of intracellular metabolism.*
Metabolic pathway of glucose, galactose, glutamine and fatty acid β-oxidation is presented to show possible metabolic changes using inhibitors of glycolysis or oxidative phosphorylation.

The rationale of the different metabolic buffers and inhibitors is that a decreased susceptibility to one of the latter may signify that the targeted pathway has a large metabolic capacity. After an initial equilibration period of 10 minutes with Tyrode's buffer, the hearts were perfused with the desired modified buffer with the metabolic fuel (glucose, galactose plus glutamine or octanoate plus malate) for 10 minutes more. After a total of 20 minute equilibration period, the inhibitors were added to the perfusate in cumulative doses: iodoacetate, 12.5 – 100 μM; rotenone, 0.25 – 10.0 μM and KCN, 0.04 - 1.0 mM. After 5 minutes at each inhibitor concentration,

experimental variables were measured. Upon the final concentration was reached, the hearts were perfused again with the metabolic buffer in the absence of inhibitors for 10 minutes more. During perfusion, all buffers were maintained at 37ºC and oxygenated with 95% oxygen plus 5% of CO_2. Figure 11 shows a representative scheme of the perfusion protocol with the different types of perfusates.

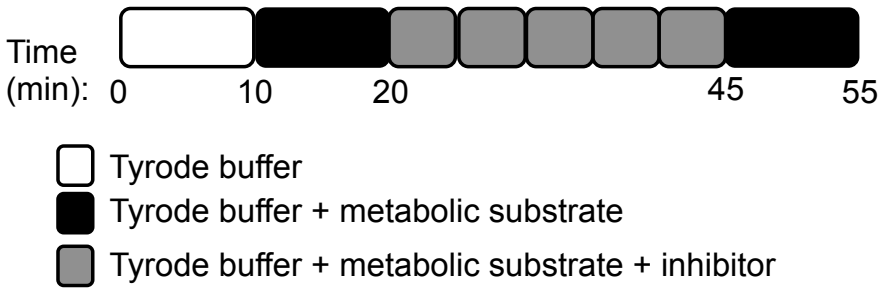

***Figure 11**. Heart perfusion scheme.*

The heart was mounted on a steel cannula placed in the perfusion column and a Tyrode´s buffer-filled latex balloon (Harvard Apparatus, Holliston, MA, USA) was placed in the left ventricle through the mitral valve and connected to a pressure transducer (Micron Instruments, Simi Valley, CA, USA) for the recording of contractility indices. Flow was adjusted by a peristaltic pump to achieve an initial perfusion pressure of ~100 mmHg and was constant for the duration of the heart perfusion.

Hearts were perfused retrogradely in the Langendorff system with the use of perfusion lines, heart chamber and thermostated reservoirs that maintained the temperature at 37ºC. Pressure signals were amplified and recorded using the software WinDaq Pro Wavelength Software (DATAQ Instruments, Inc., Akron, OH, USA). To collect and analyze the data, Origin 8.5 software (OriginLab Corporation,

Northampton, MA, USA) was used. Rate Pressure Product (RPP) was calculated by the following formula [249]:

RPP = Heart Rate (beats/min) x Peak Systolic Pressure (mmHg)

In the full set-up protocol, hearts were removed from the Langendorff system, dissected in a coronal plane and half of the tissue was immediately flash-frozen in liquid nitrogen and stored at - 80ºC until following experiments, while the other half was sustained in RNA*later* (Life Technologies, Carcavelos, Portugal).

Time controls (TC) for both acute and sub-chronic treatment related perfusions were performed by perfusing hearts with metabolic substrates without the inhibitors, for the total duration of the perfusion. Non-perfused (NP) hearts from SAL or DOX-treated Wistar rats were also quickly frozen in liquid nitrogen for follow-up experiments.

All perfusion experiments were carried out between 8 a.m. and 8 p.m.

3.5 Heart protein extraction

Protein extracts from frozen hearts were obtained by lysis and homogenizations in a glass pestle hand-held homogenizer on ice-cold 10%(w/v) lysis buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 7.4), 1 % Triton X-100, 1% DOC and 0.1% SDS, supplemented with a 10 μl/100 mg (tissue) of protease cocktail (#P8340, Sigma) and 1 tablet/100 mg (tissue) phosphatase (#04906845001, Roche) inhibitors. After tissue homogenization, the suspension was centrifuged at 14,000 x *g* for 10 min at 4ºC, with the supernatant being discarded in order to remove undesirable tissue fragments.

Lysates were resuspend in homogenization buffer and then sonicated three times for 5 sec at 60 A and stored for quantification in a -80ºC freezer.

The protein concentration was determined by the Bicinchoninic acid assay (BCA) using the commercial Pierce BCA assay kit protocol (#9981, Thermofisher Scientific), standards and unknown samples were performed in duplicates. The amount of protein amount was calculated after determining the absorbance of the dye at 545 nm in a Victor X3 plate reader (Perkin Elmer, Waltham, MA, USA).

3.6 Immunoblotting

Protein amount was quantified and diluted with a 5x concentrated Laemmli sample buffer, containing 10 mM Tris pH 6.8, 8% glycerol, 3% SDS, 0.8% β-mercaptoethanol, 0.02% bromophenol blue, to achieve a working concentration of 2.5mg/µl or 5.0 mg/µl of protein. Protein lysate was denatured at 95ºC for 5 min and separated in a vertical polyacrylamide gel electrophoresis system (Mini-PROTEAN Tetra Cell, Bio-Rad), in a 9 % or 12% SDS-polyacrylamide (SDS-PAGE) gel during 90 minutes at 120V at room temperature. A molecular weight standard was used in every running gel (#161-0374, Bio-Rad) to confirm target protein weight on the gel.

After separation of proteins by SDS-PAGE, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane previously activated, at a constant voltage of 100V during 90 minutes at 4ºC (Trans-Blot Cell, Bio-Rad). Following the transfer, membrane were incubated with blocking solution of 5% non-fat dry milk (#1706404, Bio-Rad) in Tris buffered saline with tween 20 (TBS-T) for one hour at room temperature and incubated with the primary antibodies described in table I, overnight at 4ºC, except for the anti-actin antibody which was incubated for 2

hours at room temperature.

Table I. *List of primary antibodies used in the work.*

Primary Antibody	Company/Code	Dilution	Molecular Weight
Hexokinase I	Cell Signaling #2024	1:1,000 5 % BSA	102 kDa
Hexokinase II	Cell Signaling #2867	1:1,000 5 % BSA	102 kDa
GAPDH	Cell Signaling #5174	1:1,000 5 % milk	37 kDa
PKM 1/2	Cell Signaling #3190	1:1,000 5 % BSA	60 kDa
PDH	Cell Signaling #3205	1:1,000 5 % BSA	43 kDa
LDHA	Cell Signaling #3582	1:500 5 % BSA	37 kDa
ANT	Santa Cruz sc9299	1:300 1 % BSA	33 kDa
CypD	Mitosciences #MSA04	1:10,000 1 % BSA	18 kDa
PGC- 1α	Millipore #ST1202	1:1,000 5 % BSA	113 kDa
TFAM	Santa Cruz sc23588	1:500 1 % milk	25 kDa
Beclin- 1	Cell Signaling #3495	1:750 5 % BSA	60 kDa
p62	MBL PMD045	1:750 1 % milk	62 kDa
Hsp90	Cell Signaling #4877	1:1,000 5 % BSA	90 kDa
Ubiquitin	Cell Signaling #3933	1:750 5 % BSA	10 kDa
Actin	Millipore #MAB1501	1:5,000 5 % BSA	43 kDa

Abbreviations: *GAPDH-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2-Pyruvate kinase; PDH-Pyruvate dehydrogenase; LDHA-Lactate dehydrogenase A isoenzyme; ANT-Adenine nucleotide translocator; CypD-Cyclophilin D; PGC-1α-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM-Mitochondrial transcription factor A; Hsp90 - Heat shock protein 90.*

After incubation with primary antibodies, membranes were washed with TBS-T three times, 5 minutes each and incubated with the secondary antibodies described in table II, for 1 hour at room temperature.

Table II. *List of secondary antibodies used in the work.*

Secondary Antibody	Company/Code	Dilution
Rabbit	Santa Cruz sc2007	1:5,000 1 % TBST
Mouse	Santa Cruz sc2008	1:5,000 1 % TBST
Goat	Santa Cruz sc2771	1:5,000 1 % TBST

Antibodies were prepared in 5% non-fat dry milk (Bio-Rad) in TBS-T supplemented with 0.02% sodium azide and stored at 4ºC. According to company's instructions, some antibodies were prepared in bovine serum albumin (BSA) or milk diluted in TBST.

For immunodetection, membranes were washed three times for 5 minutes each with TBST, dried and incubated with enhanced chemi-fluorescence (ECF) system (#RPN5785, GE Healthcare) during 5 minutes maximum. The ECF substrate allows the formation of fluorescence at 540 nm to 560 nm when excited at approximately 450nm and images were collected with a UVP BioSpectrum 500 Imaging System (UVP, Upland, CA, USA). Density analysis of bands was carried out with VisionWorks®LS Image Acquisition and Analysis Software (UVP, Upland, CA, USA). Ponceau staining solution after transfer was also used in membranes to confirm equal amount of protein loading.

3.7 RNA extraction

Hearts saved in RNA*later* after perfusions were used for the extraction of total RNA

using RNeasy Mini kit (#74106, Qiagen) with alterations. RNA extraction initiated with a weight of ≈ 25 mg of heart tissue suspended in 400 µl of buffer RLT. For total disruption of tissues Ultra Turrax T25 basic (IKA Works, Inc., Wilmington, NC, USA) disperser was used at 19,000 x *g* during 40 seconds. Proteinase K (#MB01902, Nzytech) was added at 10 µg/ ml into the suspension and incubated at 55ºC for 10 minutes. Once incubation was completed, the mixture was placed in QIAshedders columns (#79656, Qiagen), to which several centrifuges were performed, according to the commercial protocol of RNeasy Mini kit. RNA was eluted with 40 µl of RNase-free water (#3098, Merck Millipore) and was quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), measuring nucleic acid absorbance at 260 nm. RNA quality and purity of samples were confirmed using an Experion RNA StdSens starter kit (#700-7153, Bio-Rad) with an Experion system (Bio-Rad, Amadora, Portugal).

3.8 Primer design

Primers were designed using Beacon Designer 8.02 software (Premier Biosoft, Palo Alto, CA, USA) after obtaining nucleotide accession numbers from the database Primer-Basic Local Alignment Search Tool (Primer-BLAST). Subsequently, primers were synthesized by Sigma Aldrich (Madrid, Spain) and diluted in RNase-free water to a concentration of 100 µM upon arrival. Subsequent experiments confirmed the absence of primer non-specific products. Table III shows the sequences of primers used in the present work.

Table III. *List of primers used in the present work.*

Primer Name	Sequence	Amp. (bp)	Tm (ºC)
ANT	5′-CGGAAAGGGGCTGATATTATG-3′ 3′-ATCTGTGAACCTGTGAACTTG-5′	193	60
Hif -1α	5′-CAAGCAGCAGGAATTGGAACG-3′ 3′-CTCATCCATTGACTGCCCCA-5′	180	60
LDH	5′-GGTGAAGGGAATGTACGGCAT-3′ 3′-GAGCGACCTCATCGTCCTTC-5′	116	60
Actin	5′-AGATCAAGATCATTGCTCCTCCT-3′ 3′-ACGCAGCTCAGTAACAGTCC-5′	174	60
18S	5′-ACTCAACACGGGAAACCTC-3′ 3′-ACCAGACAAATCGCTCCAC-5′	122	60

Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase.*

3.9 Reverse transcription polymerase chain reaction (RT-PCR)

Total mRNA was converted to cDNA using the iScript cDNA Synthesis Kit (#1708891, Bio-Rad) protocol, using 0.5 μg (for acute treatment samples) and 1 μg (for sub-chronic treatment samples) in a 20 μl final volume in PCR strip tubes. Reactions were performed in a Bio-Rad S1000 thermal cycler (Bio-Rad, Amadora, Portugal) following the running single cycle proposed in the protocol.

Transcript levels were quantified in real-time PCR using SsoFast EvaGreen Supermix (#172-5201, Bio-Rad), with amplification and quantification of primers products executed in a CFX96 Real-time PCR detection system (Bio-Rad, Amadora, Portugal), following the cycling conditions: 1 cycle of 30 seconds at 95ºC; 40 cycles of 5 seconds

at 95ºC; 40 cycles of 5 seconds at 60ºC, acquisition of a single-point fluorescence; 1 cycle of 5 seconds from 65ºC to 95ºC, ended by a melting curve program. All RT-PCR steps were performed with Multiplate low-profile 96-well unskirted PCR plates (#MLL-9601, Bio-Rad) and sealed with Microseal "B" adhesive seal (#223-9444, Bio-Rad) following a plate scheme with duplicates of samples, a non-template control (NTC) and a negative control where cDNA template was absent (NRT), to safeguard contaminations. Standard curves were also performed for the respective transcripts, which cover the range in the sample unknowns, this allowed calculate the efficiency of the reaction. A preparation of standards curves was executed with HotStartTaq Master Mix kit (#203443, Qiagen), using a simple PCR reaction with forward and reverse primers, a cDNA control sample and the master mix. After that, the product was purified using MinElute PCR Purification kit (#28004, Qiagen) and the protocol was followed until nucleic acid quantification in NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA).

Standard stock solution was prepared with $5x10^9$ copies/ml using the equation:

$$\text{Number of copies} = \frac{\text{Concentration standard x Avogadro's number}}{\text{Length amplicon x 650}}$$

3.10 mRNA transcripts expression analysis

Levels of selected mRNA were analysed wih CFX Manager Software 3.0 (Bio-Rad, Amadora, Portugal). During the analyses, references genes, such as β-actin and 18S, were used to minimize loading differences or variations between each sample,

helping in the normalization of the expression level of the gene of interest. In the present study, we only used β-actin as reference genes.

Normalized expression was calculated using the "delta-delta C_t" ($\Delta\Delta C_t$), this measure results from a calculation model based on multiple samples and a single reference gene. Parameters such as crossed-point value of the sample and the efficiency of reaction are imperative to verify the value of the RT-PCR reaction, these factors are calculated on basis of a linear regression slope of the standards curves.

3.11 Statistical Analysis

Data obtained for this thesis was analyzed using the software Graph Pad Prism version 6.0c for Macintosh (GraphPad Software, Inc., San Diego, CA, USA). All data were accessed for normality with Kolmogorov-Smirnov test. Data are expressed as mean ± SEM for the number of experiments/animals indicated in the legends of the figures. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test. Student's t-test was used when only two conditions were compared. Significance was accepted when p value < 0.05 was obtained.

Chapter 4: Results and Discussion

4.1. Doxorubicin Treatment – Acute treatment protocol

In the present chapter, we analyzed how DOX acute treatment affects cardiac hemodynamic behavior with specific metabolic conditions under the absence or presence of iodoacetate, an inhibitor of glycolysis, which irreversibly inhibits the glycolytic enzyme GAPDH [246], rotenone [247] and KCN [248] both inhibitors of the mitochondria respiratory chain, namely by acting on complex I and IV, after hearts were collected and proteins and mRNA transcripts content were evaluated.

4.1.1 Consequences of doxorubicin acute treatment on Wistar rat body weight

Saline (n=46) and DOX-treated (n=50) animals were weighted before the single injection of DOX and at the time of animal sacrifice. The results show that there were no differences in the initial or final body weight after the administration of SAL or DOX (Fig. 12).

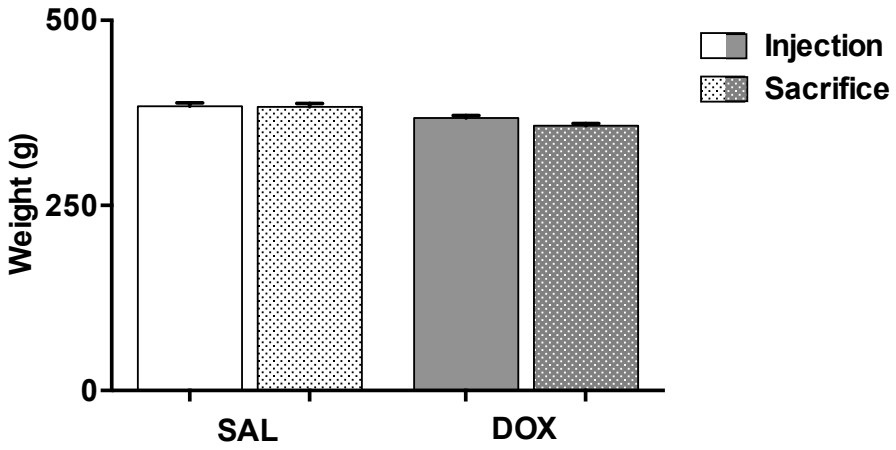

***Figure 12**. Body weight of saline (SAL) and doxorubicin (DOX) acutely treated animals. Student´s t-test (DOX vs. SAL for the same time point), data are mean ± SEM, SAL n= 46, DOX n= 50.*

We also observed that the heart over body mass ratio did not present any difference (Fig. 13A); however, significant differences were found between treatments regarding heart mass, with a reduced heart mass in DOX-treated animals, (1.075 g ± 0.01) comparing with the SAL group (1.141 g ± 0.02) (Fig. 13B).

When hearts were observed, none of them showed any apparent size, color or morphological alterations when comparing both groups.

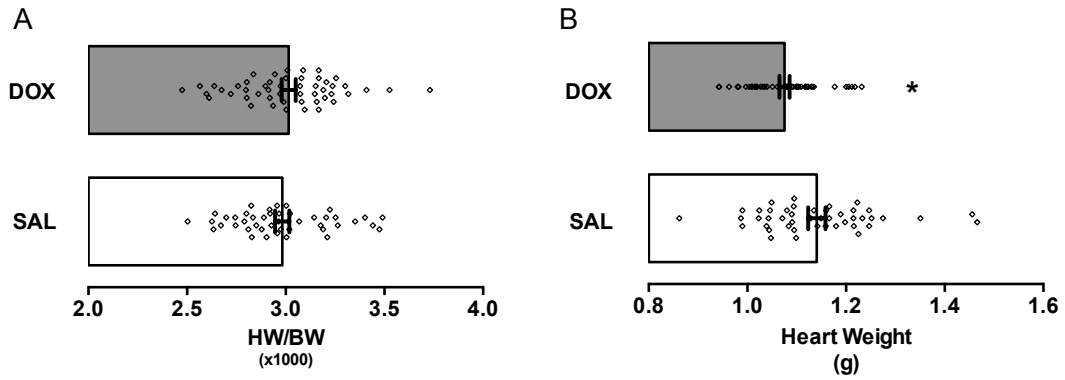

Figure 13. *Heart weight / body weight ratio (A) and heart weight (B) of saline (SAL) and doxorubicin (DOX) acutely treated animals. Student´s t-test, data are mean ± SEM, SAL n= 46, DOX n= 50. * p < 0.05 SAL vs. DOX*

4.1.2 Heart hemodynamic rates are disturbed under metabolic inhibition- role of substrate

The Langendorff perfusion system allowed us to manipulate the metabolic activity of hearts from both SAL and DOX treatment group, by using glucose, galactose plus glutamine and octanoate plus malate as fuels. For each perfusion condition, three different inhibitors were added separately, iodoacetate (12.5 to 100 μM), rotenone (0.25 to 10.0 μM) and potassium cyanide (KCN) (0.04 to 1.0 mM).

4.1.2.1 Time controls for SAL and DOX hearts show similar behavior

As a necessary perfusion control, we performed a time control experiment, representing the total time of heart perfusion with the three fuels, glucose, galactose plus glutamine, and octanoate plus malate (Fig. 14). The results with glucose showed that hearts from both treatments differ in heart rate (Fig. 14A) during the first 30 minutes of perfusion with interestingly, DOX-treated group showing a higher heart rate. When measuring the RPP (Fig. 14B), only at 30 minutes perfusion the SAL group showed a significantly higher value than the DOX-treated group.

Using octanoate plus malate as substrate, a decreased heart rate at 55 minutes of perfusion was observed in the DOX group compared with SAL hearts (Fig. 14E), without any consequences on the RPP (Fig. 14F). No differences were found when comparing both groups with galactose plus glutamine.

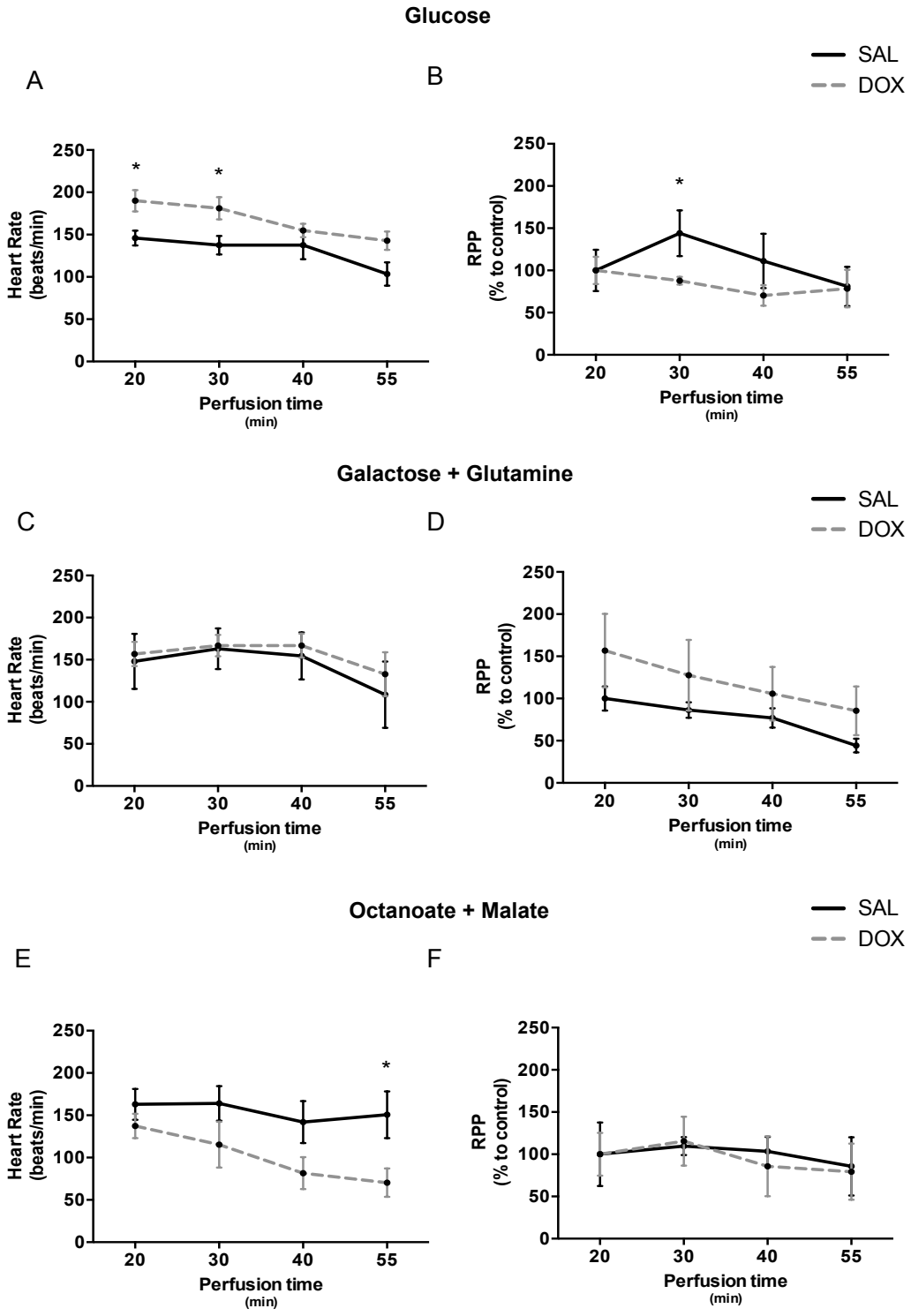

Figure 14. *Time control perfusion of SAL and DOX hearts with glucose (A and B, n=5-6), galactose plus glutamine (C and D, n=5-6) and octanoate plus malate (E and F, n=5-6).*
*No differences were found between groups. Comparisons between SAL and DOX groups for the same perfusion time were performed using a Student´s t-test. Comparisons of perfusion time effects vs. control (20 min) were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, * $p < 0.05$ SAL vs. DOX.*

4.1.2.2 Effect of acute DOX treatment on heart hemodynamic behavior under metabolic inhibition: glucose as fuel source

Using glucose as metabolic supply, different responses were obtained when hearts were titrated with the inhibitors. When the titration was performed with iodoacetate, the heart rate (Fig. 15A) and the RPP (Fig. 15B) decreased as the concentration of iodoacetate increased, with a difference found at 75 and 100 μM when compared with the initial absence of inhibitor (indicated as 0 in graph), which was used to equilibrate the hearts. The differences were found in both SAL and in DOX-treated hearts. Both groups did not recover at the end of the perfusion when iodoacetate was removed from the glucose buffer following 10 min of perfusion, this period designated as recovery phase. The perfusion with rotenone or KCN did not show any significant differences between treatments or during the perfusion with increasing inhibitor concentrations (Fig. 15). No differences between treatment groups were identified for any of the perfusion conditions tested.

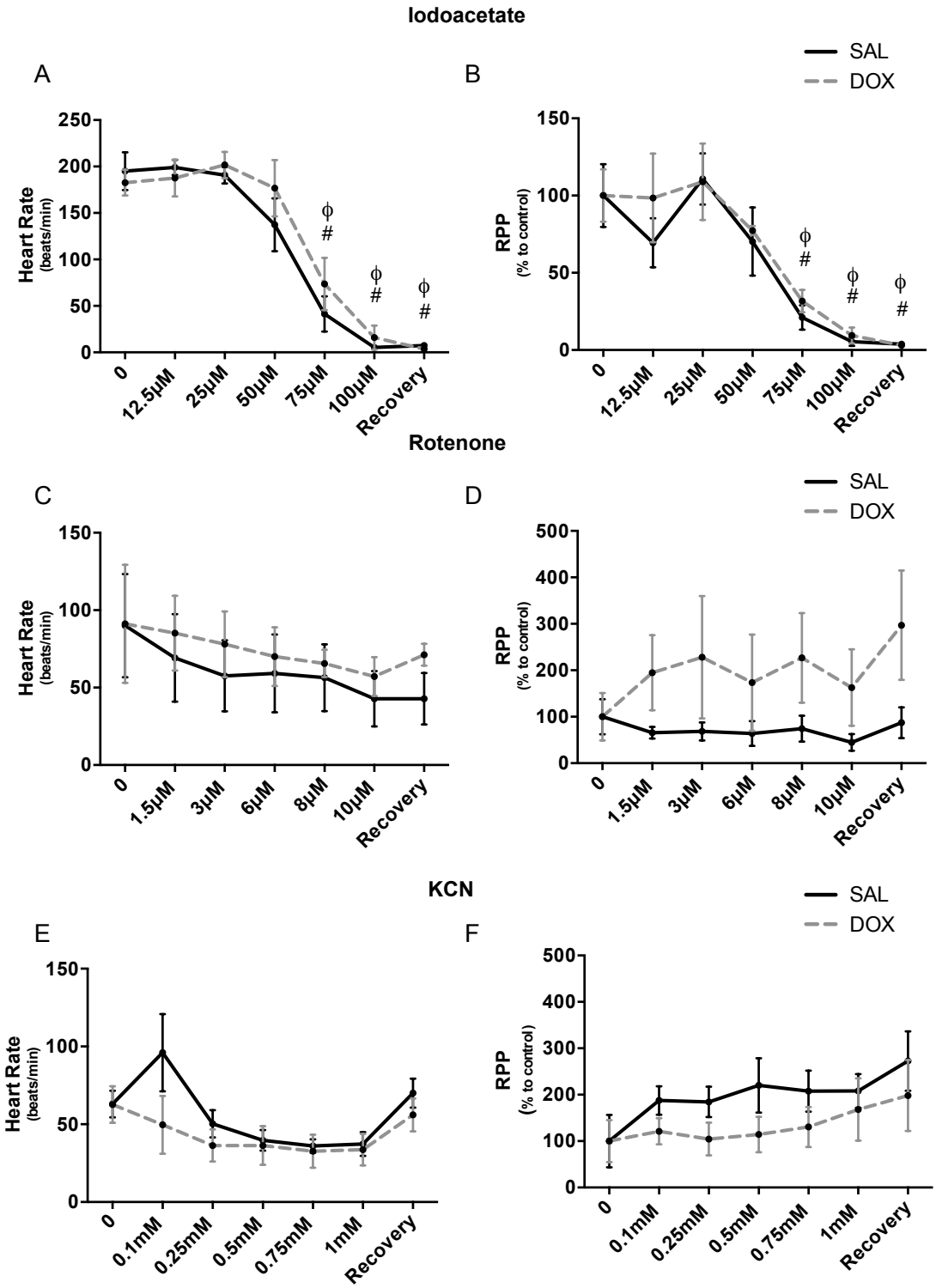

Figure 15. *Heart perfusion with glucose titration with iodoacetate (A and B, n=5-6), rotenone (C and D, n=5-6) and KCN (E and F, n=5-6).*

Initial perfusion is represented in the graph as "0", and recovery phase in the absence of inhibitors as "Recovery". Comparisons between SAL and DOX groups for the same perfusion condition were performed using a Student´s t-test. Comparisons of inhibitor concentration vs. initial perfusion (indicated as 0) were performed with one-way ANOVA, followed by the Bonferroni post-test. # $p < 0.05$ SAL vs. 0 , Φ $p < 0.05$ DOX vs. 0., data are mean ± SEM.

4.1.2.3 Effect of acute DOX treatment on heart hemodynamic behavior under metabolic inhibition: galactose plus glutamine as fuel sources

When hearts were perfused with galactose plus glutamine (GG), to stimulate the TCA cycle pathway and decrease the contribution from glycolysis for ATP production, again inhibitor-related differences were found. Iodoacetate decreased both heart rate (Fig. 16A) and RPP (Fig. 16B). In the SAL group, a significant decrease of heart rate in the presence of 50, 75 and 100 μM iodoacetate was found, leading to a low heart rate at the end of the re-perfusion without inhibitor. In the DOX group, the decrease is significant for 75 and 100 μM, without any final recovery observed (Fig. 16A). Significant differences between treatments were observed with 100 μM of iodoacetate and at the recovery phase (Fig. 16A). In the RPP graph (Fig. 16B), the SAL group was interestingly the most affected with iodoacetate, showing a significant decrease in that value for concentrations of 25 μM and above. The DOX group did not show any differences between the initial perfusion and the addition of iodoacetate. However, for 25, 50 and 75 μM iodoacetate, a significant difference between treatments was observed, as well as during the recovery phase, with the DOX group being less affected by the inhibitor.

The use of rotenone affected cardiac performance. A significant decline of heart rate with 1, 1.5 and 2 μM rotenone in the DOX group when compared with initial perfusion was observed (Fig. 16C). The SAL group showed a decrease of heart rate with 2 μM, the maximum rotenone concentration used. However, the SAL group

recovered its function after washing the inhibitor, as opposed to the DOX group, which showed a decline in function during the recovery phase. During the perfusion with rotenone, the RPP (Fig. 16D) suffered a decline with both treatments; however, only the DOX group showed differences during the perfusion with 1.5 μM rotenone and at the recovery phase.

Clearly, a titration of the perfused hearts with KCN caused a higher disturbance that any other inhibitor with this metabolic fuel. In the DOX group, almost every single KCN concentration used (from 0.1 μM to 0.5 μM) caused a significant decrease in the heart rate (Fig. 16E) and in the RPP (Fig. 16F) resulting in an inability to recover the normal function in both groups. The same appears to occur in the SAL group, with a decrease of both parameters by KCN in a dose-dependent manner, also revealing an inability to recover. Regarding the RPP data (Fig. 16F), a significant difference was found for 0.05, 0.1 and 0.2 mM KCN for both treatment groups. Generally, with iodoacetate and KCN, the hearts from the SAL group were more affected that the DOX counterparts.

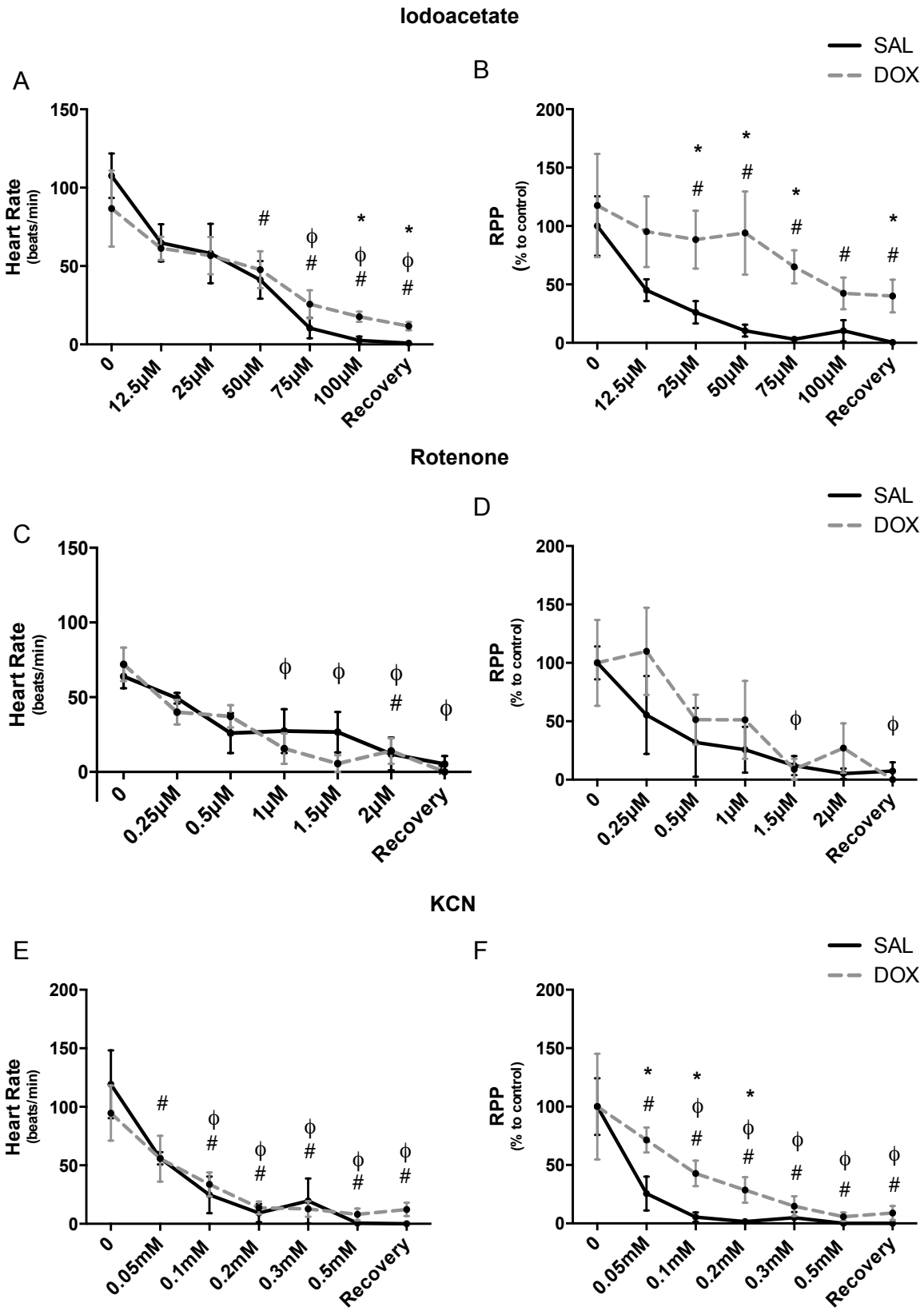

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Figure 16. *Heart perfusion with galactose plus glutamine titration with iodoacetate (A and B, n=4-6), rotenone (C and D, n=4-6) and KCN (E and F, n=4-6).*
*Initial perfusion is represented in the graph as "0", and recovery phase in the absence of inhibitors as "Recovery". Comparisons between SAL and DOX groups for the same perfusion condition were performed using a Student´s t-test. Comparisons of inhibitor concentration vs. initial perfusion (indicated as 0) were performed with one-way ANOVA, followed by the Bonferroni post-test. # $p < 0.05$ SAL vs. 0 , Φ $p < 0.05$ DOX vs. 0 , * $p < 0.05$ SAL vs. DOX, data are mean ± SEM.*

4.1.2.4 Effect of acute DOX treatment on heart hemodynamic behavior under metabolic inhibition: octanoate plus malate as fuel sources

Figure 17 shows heart perfusion data using octanoate plus malate (OM) as the metabolic supply, activating a predominant fatty β-acid oxidation pathway in the generation of energy. Starting with iodoacetate titration during perfusion, a decrease in both groups in the heart rate (Fig. 17A) and in the RPP (Fig. 17B) was found. However, only DOX presented a significant decrease in the heart rate with 100 μM iodoacetate, without any recovery in the absence of the inhibitor. For the concentrations of 25, 50, 75 and 100 μM of iodoacetate, DOX-treated hearts showed a significant decline in RPP (Fig. 17B), while the SAL group was inhibited for concentrations of 75 and 100 μM. Both treatment groups did not recover once iodoacetate was removed from the perfusion.

The use of rotenone and KCN severely interfered with cardiac function, with the RPP (Fig. 17D and F, respectively) showing a decline for both SAL and DOX groups and with all concentrations used. Regarding heart beat (Fig. 17C) concentrations of 0.75, 1 and 1.5 μM rotenone caused a decrease in the DOX group, while for the SAL group,

the decline only occurred for 1.5 μM rotenone, with both groups failing in the recovery phase.

With KCN as the inhibitor (Fig. 17E and F), both heart rate and RPP were significantly decreased for all concentrations used, except for the lowest concentration (0.04 mM) in the SAL group. Differences between treatments were observed in heart rate (Fig. 17E) with concentrations of KCN of 0.08 and 0.1 mM, without any effect on the RPP.

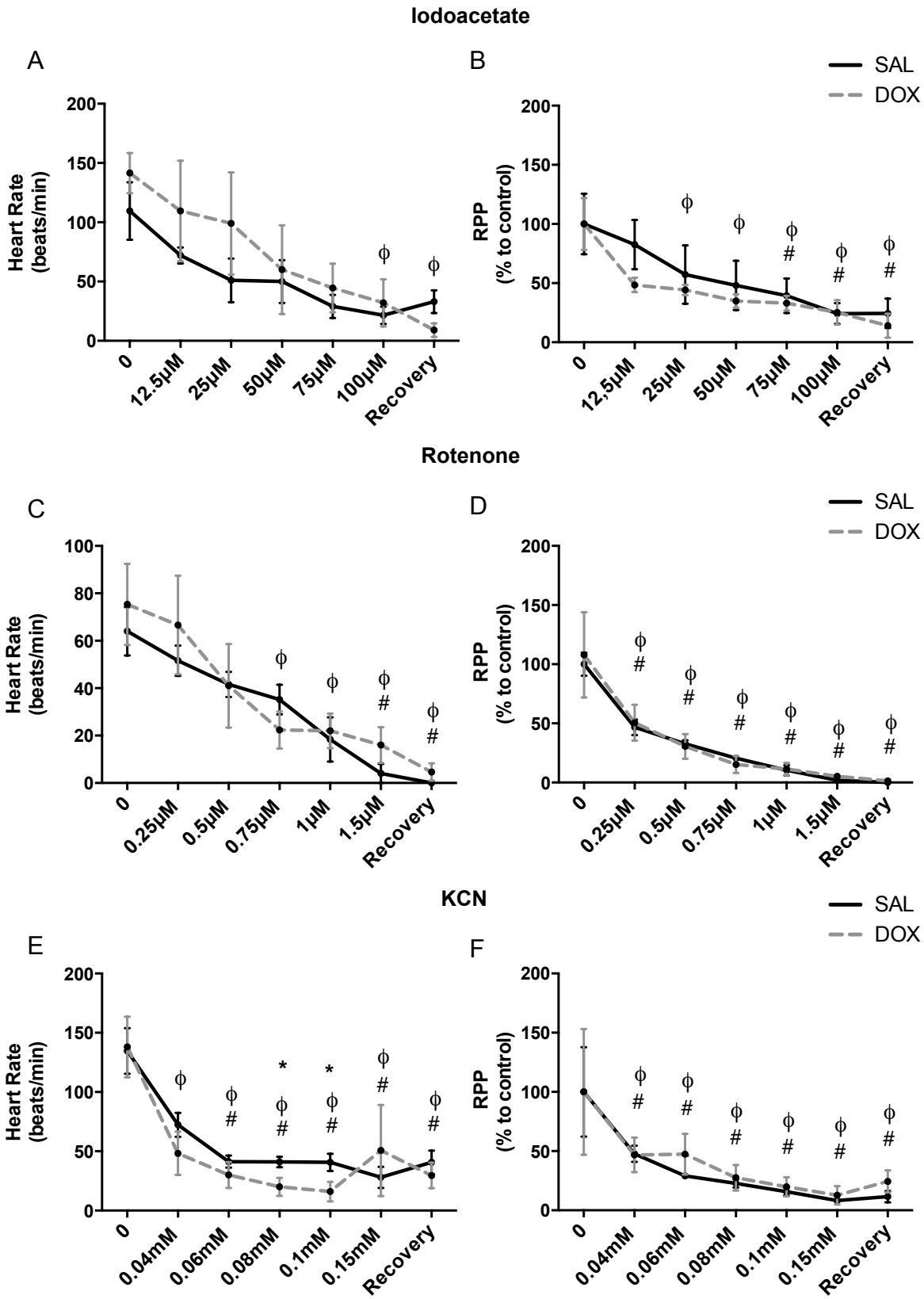

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Figure 17. *Heart perfusion with octanoate plus malate titration with iodoacetate (A and B, n=4-6), rotenone (C and D, n=4-6) and KCN (E and F, n=4-6).*
*Initial perfusion is represented in the graph as "0", and recovery phase in the absence of inhibitors as "Recovery". Comparisons between SAL and DOX groups for the same perfusion condition were performed using a Student´s t-test. Comparisons of inhibitor concentration vs. initial perfusion (indicated as 0) were performed with one-way ANOVA, followed by the Bonferroni post-test. # $p < 0.05$ SAL vs. 0 , Φ $p < 0.05$ DOX vs. 0, * $p < 0.05$ SAL vs. DOX, data are mean ± SEM.*

4.1.3 Protein alterations in perfused hearts in SAL and DOX-acutely treated rats: role of substrate and metabolic inhibition

Metabolic modulation in heart is frequent in several pathologies, such as during ischemia, cardiomyopathies or even during the drug-induced toxicity including with DOX [12,250].

After the perfusion with inhibitors followed by the recovery time, SAL and DOX-treated hearts as well as non-perfused hearts from both groups, were collected and selected proteins involved in mitochondrial function, cell metabolism and autophagy were semi-quantified by Western blotting. The rational is that not only the treatment (SAL vs. DOX) can impact protein expression but also the impact of metabolic remodeling and/or metabolic inhibition during perfusion can alter the content of the referred proteins.

4.1.3.1 Non-Perfused (NP) SAL and DOX acutely treated hearts

We initially probed proteins related the glycolytic pathway in animals treated with SAL or DOX, but without the perfusion protocol, the so-called non-perfused (NP) control hearts.

In this work, several glycolytic proteins were evaluated including GAPDH, pyruvate kinase (PKM 1/2), PDH and lactate dehydrogenase A isoenzyme (LDHA). Hexokinase catalyzes the conversion of glucose to glucose-6-phosphate, with hexokinases I, II being associated with the outer mitochondrial membrane and critical for maintaining an elevated rate of aerobic glycolysis [251]. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the phosphorylation of glyceraldehyde-3-phosphate during glycolysis [252], while PKM 1/2 catalyzes the conversion of phosphoenolpyruvate to pyruvate. Pyruvate kinase isoform 1 is expressed in adult tissues while isoform 2 is expressed during embryonic development [253]. Pyruvate dehydrogenase catalyzes the condensation of pyruvate and CoA into acetyl-CoA, which follows the TCA cycle [254]. Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and NADH to lactate and NAD+, recycling NADH generated in glycolysis to NAD+, which is reduced again during glycolysis. The major LDH isoform found in muscle cells is A isozyme [11].

Comparing both SAL and DOX treatments, no differences were found in proteins analyzed (Fig. 18).

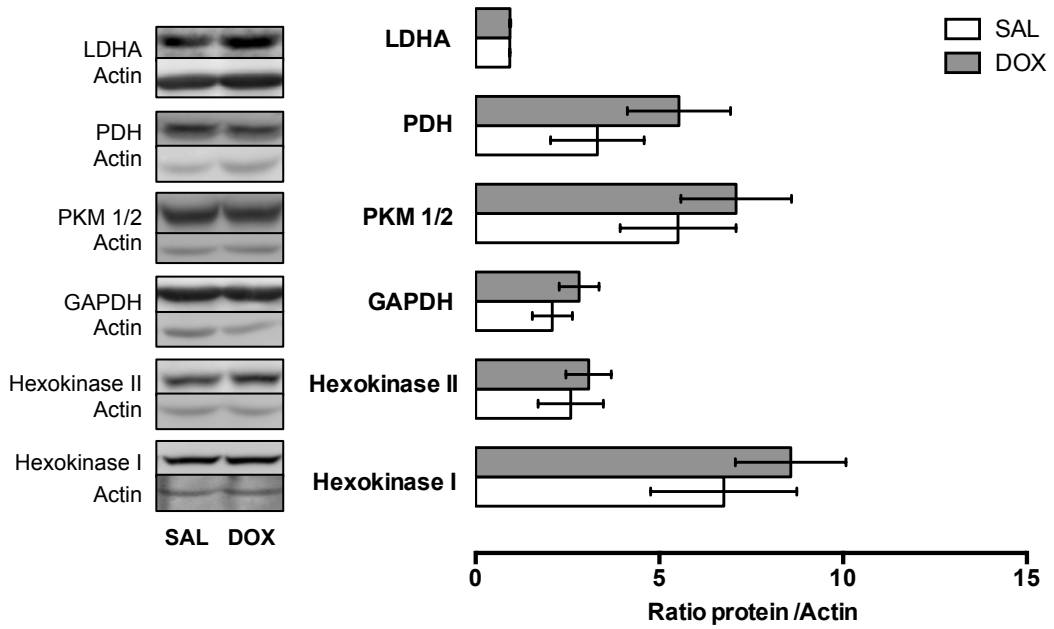

Figure 18. *Amount of protein related to the glycolytic pathway was measured by Western blotting.*

Representative Western blot for the mentioned proteins together with their respective loading control. Student´s t-test, data are mean ± SEM, (n=5-6). No differences were found between groups.

Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa).*

Other proteins relevant for mitochondrial function were also investigated, such as ANT, CypD, PGC-1α, TFAM, Beclin-1, p62, Hsp90 and ubiquitin. Mitochondrial proteins such as ANT or CypD, are proposed regulators of the permeability transition pore complex (PTP) located in the mitochondrial inner membranes [94,95,255], participating also in the regulation of apoptosis [101]. The ANT is critical for the exchange of ATP with ADP in the inner mitochondrial membrane [256] while CypD is a matrix chaperone [257]. PPARγ coactivator-1α interacts with a diverse array of transcription factors regulating cell processes important for adaptive thermogenesis and energy metabolism, including the related functions of glucose

uptake, gluconeogenesis, insulin secretion, and mitochondrial biogenesis [258]. The TFAM is a transcriptional factor for mitochondrial DNA (mtDNA), regulating mtDNA transcription and its maintenance, improving ATP production [259]. Hsp90 is a molecular chaperone which is constitutively expressed under normal conditions to maintain protein homeostasis, being induced upon environmental stress, and interacting with unfolded proteins to prevent irreversible aggregation [260]. Beclin- 1 and p62 are proteins related with the autophagic process, beclin-1 participates in the regulation of autophagy and is required for initiating the formation of the autophagosome [261], p62 is also involved in cell signaling, oxidative stress and autophagy, through interaction with ubiquitin, providing a scaffold for several signaling proteins and triggering degradation of proteins through the proteasome [262]. Ubiquitin is a conserved polypeptide unit that plays an important role in the ubiquitin-proteasome pathway, it can be linked to many cellular proteins by the ubiquitination process, which targets proteins for degradation by the proteasome [263].

By analyzing the proteins described above by Western blotting (Fig. 19) differences between treatments were found in proteins related with mitochondrial biogenesis and autophagy. Both PGC- 1α and TFAM, two proteins that are related with mitochondrial biogenesis decreased in DOX hearts when comparing with SAL hearts. The autophagy proteins Beclin-1 and p62, showed an increase in the DOX group when compared with the SAL group.

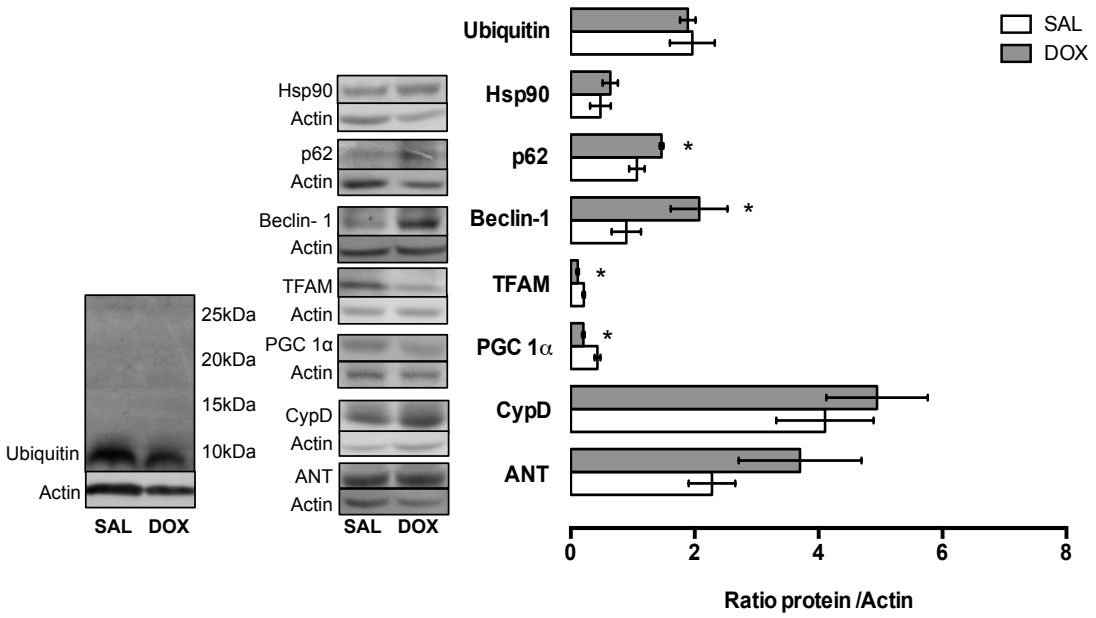

Figure 19. *Protein amount on non-perfused hearts from both treatment groups was measured by Western blotting.*

*For ubiquitin quantification, a larger area was quantified for each group. Representative Western blot for the mentioned proteins together with their respective loading control. Student´s t-test, data are mean ± SEM, (n=5-6), * p < 0.05 SAL vs. DOX.*

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa).*

4.1.3.2 Protein alterations in hearts perfused with glucose: role of substrate and metabolic inhibition

We next performed protein analyses in SAL or DOX-perfused hearts with inhibitors with a glucose-based perfusion buffer. After the recovery period, hearts were collected and proteins extracted and analyzed by Western blotting. Regarding the same glycolytic proteins described previously, no alterations between SAL or DOX groups were found. However, when we compare hearts from TC with hearts perfused in the presence of inhibitor, significant differences in several proteins were

observed (Fig. 20). Regarding Hexokinase II and LDHA, a decrease in the two proteins was observed in both SAL and DOX group. The same happened with GAPDH, but only after titration with iodoacetate or KCN in the DOX hearts, while SAL hearts decreased in the presence of all three inhibitors. Interestingly, PDH protein amount increased in SAL and DOX hearts when iodoacetate is present and only in DOX hearts when rotenone was present in the perfusate, while PKM 1/2 also increased with iodoacetate titration in SAL and DOX hearts (Fig. 20).

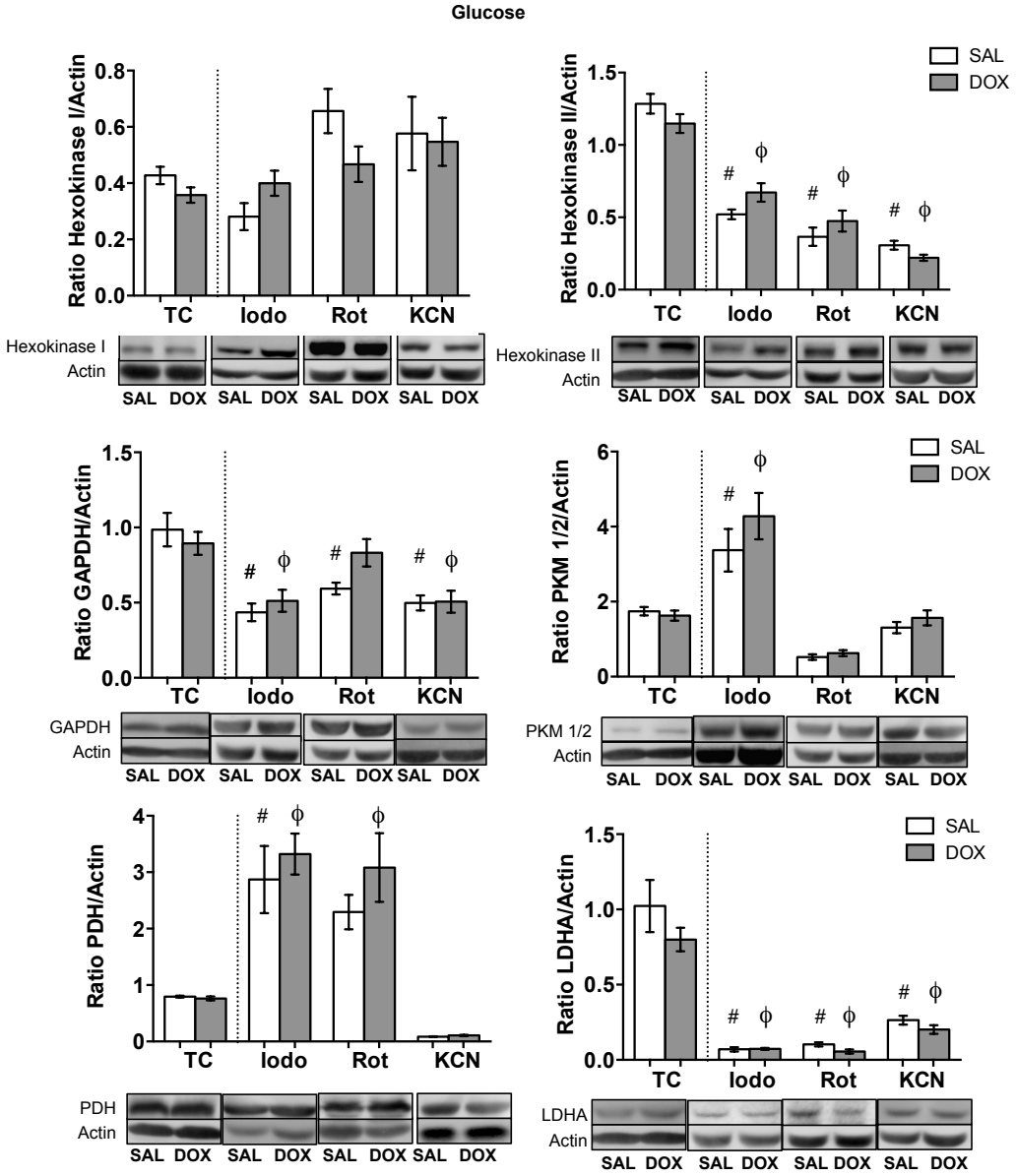

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Figure 20. *Proteins involved in the glycolytic pathway probed in glucose perfused hearts from SAL or DOX-treated rats.*

Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX.

Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

When assessing others proteins, p62 was significantly increased in DOX hearts perfused with iodoacetate and rotenone comparing with SAL hearts, while no other differences were found in any other protein, with the exception of Hsp90 which was increased in DOX hearts after the titration with rotenone (Fig. 21). By comparing differences in TC hearts with hearts perfused with inhibitor, we observed that SAL and DOX hearts perfused with KCN had increased proteins such as the ANT and CypD. Proteins PGC-1α, TFAM, Hsp90 and p62 protein showed a reduction in both SAL and DOX groups when all the three inhibitors were used. Observing ubiquitin proteins, a decrease was detected in SAL hearts perfused with rotenone comparing with TC SAL hearts. The presence of iodoacetate also decreased Beclin-1 in SAL and DOX hearts after the titration when compared with the TC condition (Fig.21).

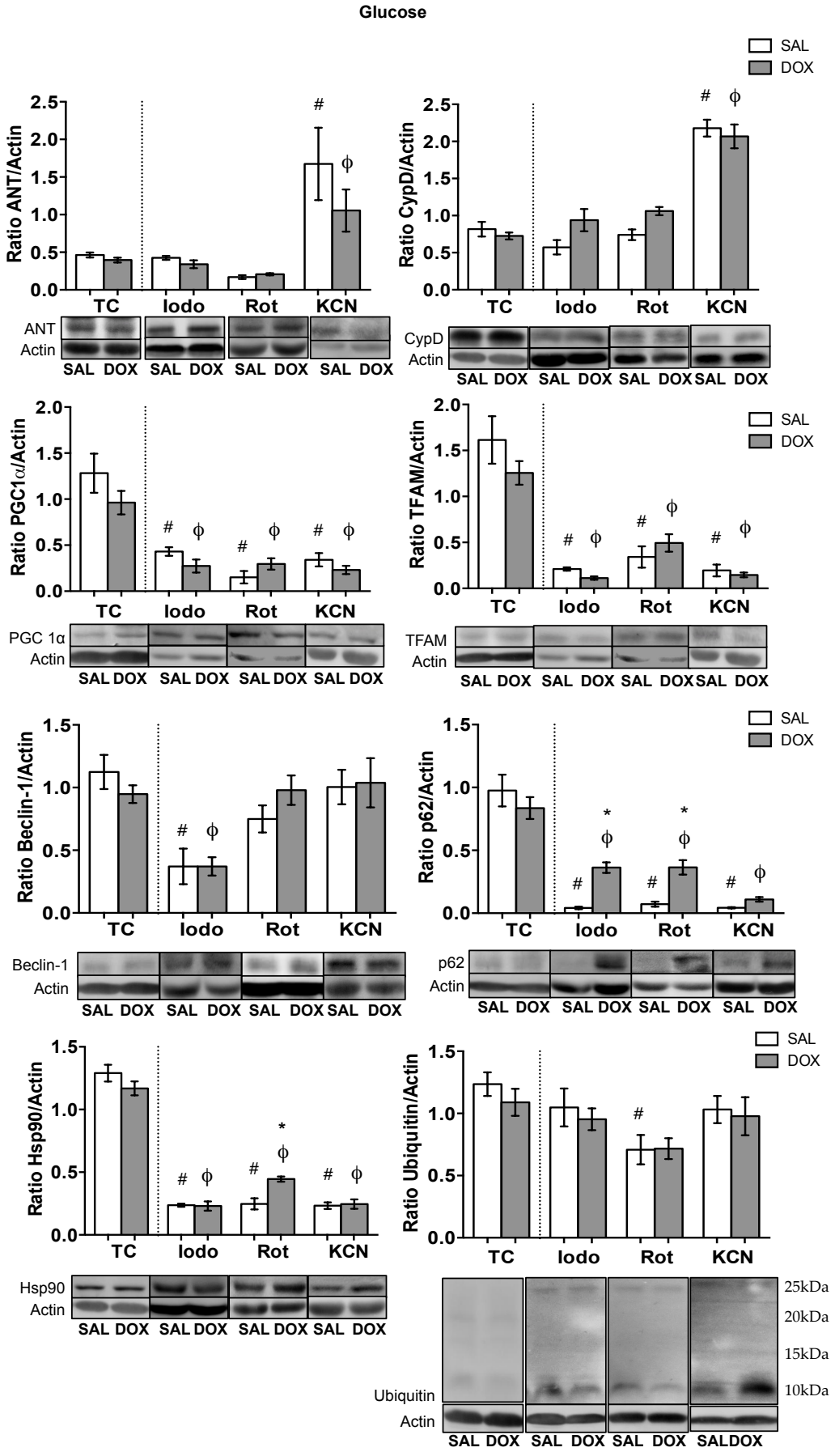

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Figure 21. *Proteins involved in the mitochondria events probed in glucose perfused hearts from SAL or DOX-treated rats.*

Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. For ubiquitin quantification, a larger area was quantified for each group. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. . Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX.

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.3.3 Protein alterations in hearts perfused with galactose plus glutamine: role of substrate and metabolic inhibition

We performed the same protocol described before on hearts from both treatment groups and perfused with galactose plus glutamine, followed by the three inhibitors in distinct experiments. The results (Fig. 22) show that hearts from the DOX-treated group had decreased Hexokinase I, Hexokinase II and PDH after titration with iodoacetate, when compared with the SAL group. Comparing hearts from TC group and after inhibition titration, we observed that the presence of the three inhibitors during perfusion decrease Hexokinase II, PKM 1/2 and LDHA on DOX hearts, while on SAL hearts only PKM 1/2 and LDHA suffered a decrease for all inhibitors, although that for hexokinase II, a decrease was only detected in SAL hearts perfused with KCN. Hearts DOX-treated perfused with rotenone showed decreased

Hexokinase I and PDH protein, when comparing with the respective TC heart, SAL hearts also decrease PDH protein amount when rotenone was used. For GAPDH, iodoacetate was the only inhibitor that increased its amount in both hearts, when comparing with the TC group (Fig. 22).

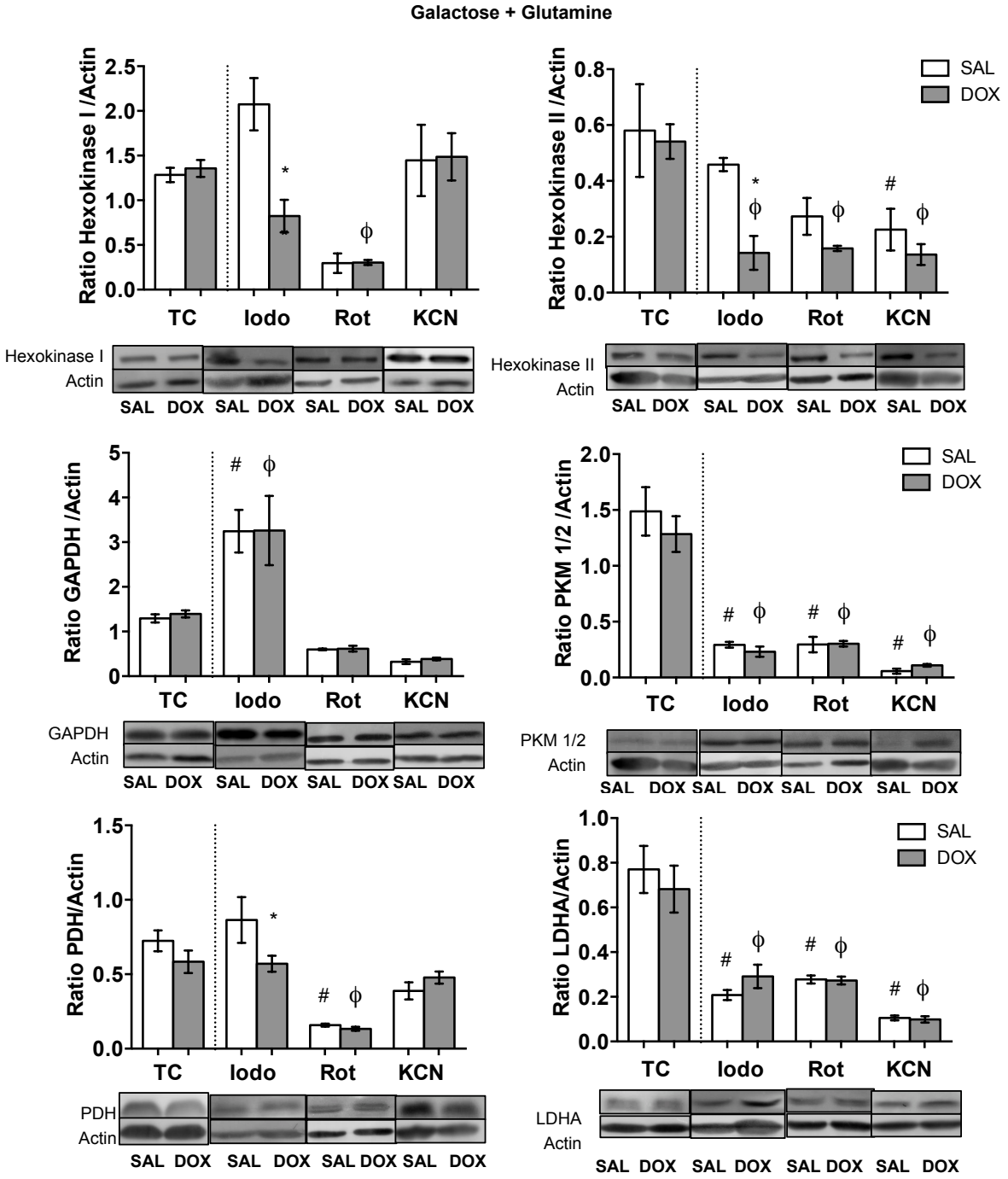

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***Figure* 22.** *Proteins involved in the glycolytic pathway probed in galactose plus glutamine perfused hearts from SAL or DOX-treated rats.*
*Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed by using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX, * $p < 0.05$ SAL vs. DOX.*
Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

Beclin-1, implicated in the autophagy process, increased in DOX hearts after perfusion with iodoacetate. However, ubiquitination of proteins decreased after iodoacetate titration in the DOX group, when compared with the SAL group. Also, a decrease in the amount of ANT, TFAM and Hsp90 proteins was measured in DOX hearts perfused with iodoacetate, when compared with the SAL group (Fig. 23). When comparing proteins from TC heart with hearts perfused with inhibitors, we observed a decrease in DOX hearts with all three inhibitors, such as ANT, CypD, PGC-1α, TFAM, p62 and Hsp90, meanwhile SAL hearts also showed decreased CypD, TFAM, p62 and Hsp90 for all the inhibitors. Protein such as ANT increased in SAL hearts when iodoacetate was present in perfusate, and with rotenone and KCN shows the opposite. By evaluating ubiquitinated protein amount, an increase in SAL and DOX hearts perfused with iodoacetate and a decrease when rotenone was added as the inhibitor was observed when comparing with hearts perfused in the absence

of inhibitors. Regarding Beclin-1, iodoacetate caused an increase in both SAL and DOX hearts, while KCN resulted in a decrease in the same protein, comparing with hearts of TC group (Fig. 23).

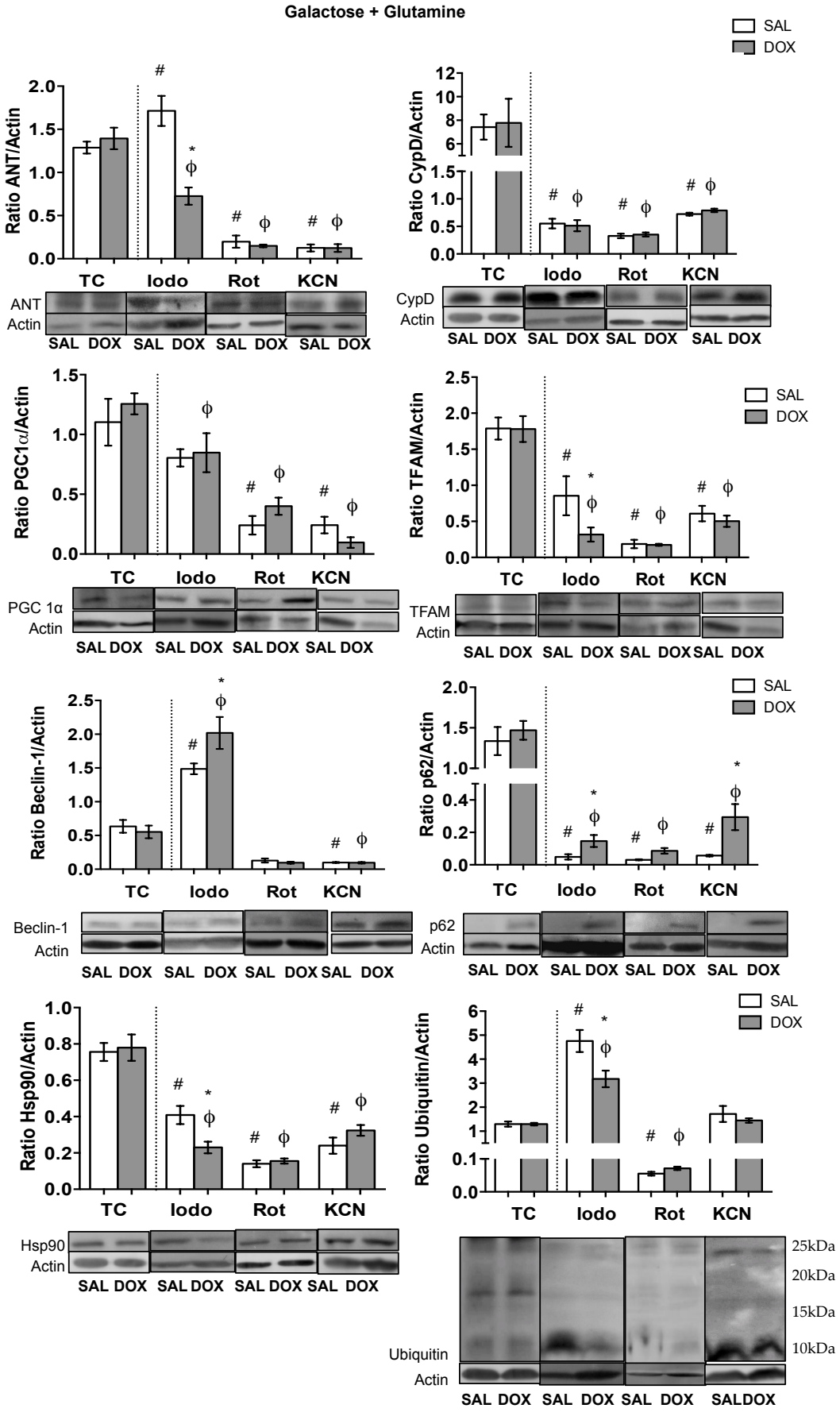

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Figure 23. *Alteration in selected proteins in galactose plus glutamine perfused hearts from SAL or DOX-treated rats.*

*Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. For ubiquitin quantification, a larger area was quantified for each group. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed by using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX, * $p < 0.05$ SAL vs. DOX.*

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.3.4 Protein alterations in hearts perfused with octanoate plus malate: role of substrate and metabolic inhibition

When hearts were perfused with octanoate plus malate, glycolytic proteins analyzed showed no alterations between SAL and DOX groups (Fig. 24). However, Hexokinase I, Hexokinase II and LDHA decreased for all the inhibitors used in DOX group, when comparing with the TC DOX-treated hearts, for SAL hearts the same effect happens, excepting for Hexokinase I with iodoacetate. A decrease was also observed for PKM 1/2 for both SAL and DOX hearts, but only in the presence of KCN. The presence of KCN on the perfusate resulted in an increase of GAPDH in the DOX group (Fig. 24).

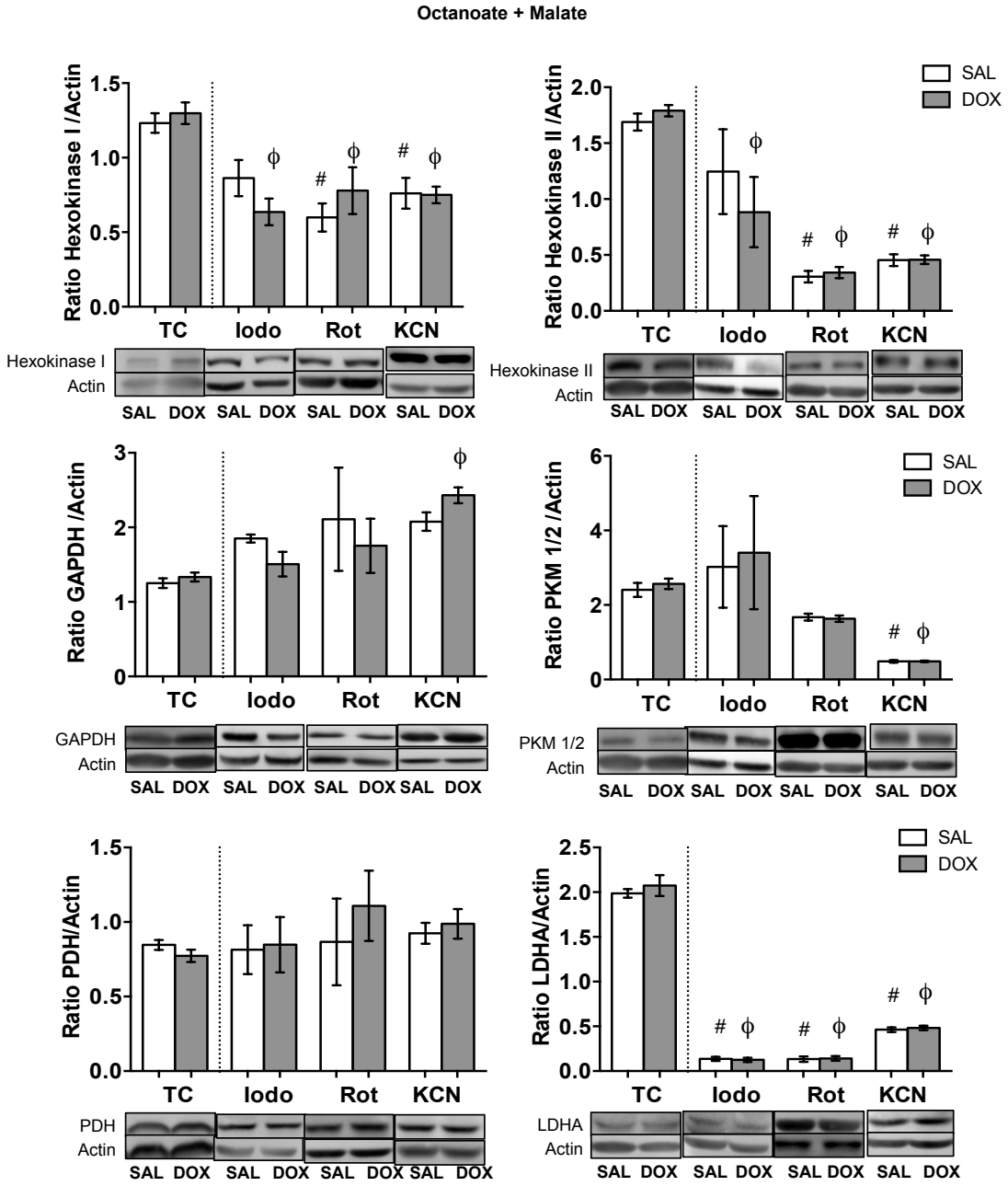

Figure 24. *Proteins involved in the glycolytic pathway probed in octanoate plus malate perfused hearts from SAL or DOX-treated rats.*

Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC

DOX vs. inhibitor DOX.
Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

When evaluating p62, a significant increase in the band intensity was observed in DOX hearts in the presence of the three inhibitors, when comparing with SAL hearts (Fig. 25). Differences between hearts from TC group and hearts perfused with inhibitors, revealed a decrease of ANT, PGC-1α, TFAM, Beclin-1 and p62 after titration with all three inhibitors for both SAL and DOX hearts. The same decreasing effect occurs in DOX hearts with Hsp90, but only after iodoacetate and KCN titration, while in SAL hearts all the inhibitors decrease Hsp90 protein amount. Observing CypD, KCN was the only inhibitor that increased protein amount in both SAL and DOX hearts, when comparing with respective TC hearts. Ubiquitin labeling increased in SAL and DOX hearts perfused with iodoacetate when compared with TC (Fig. 25).

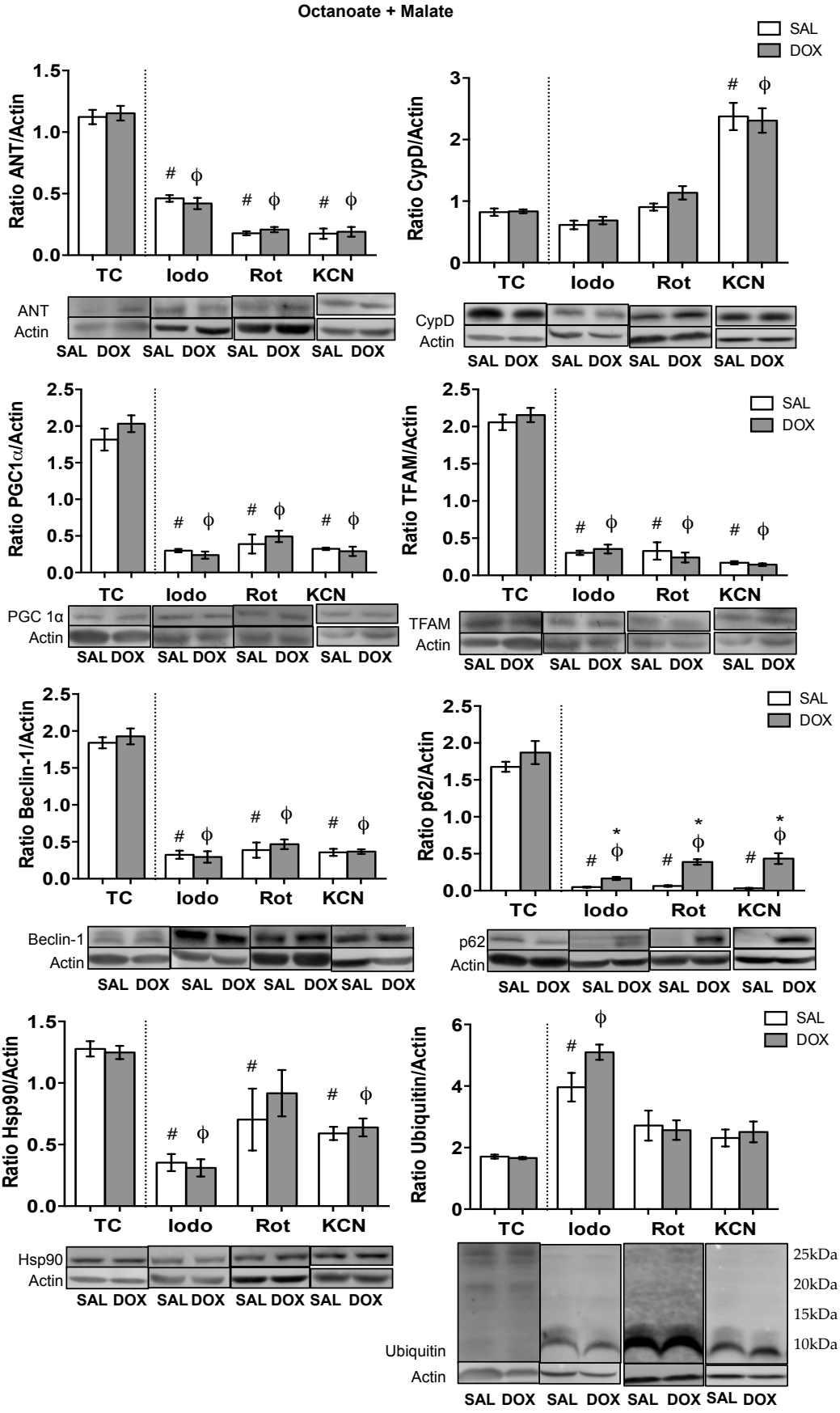

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Figure 25. *Alteration in selected proteins in octanoate plus malate perfused hearts from SAL or DOX-treated rats.*

*Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. For ubiquitin quantification, a larger area was quantified for each group. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX, * $p < 0.05$ SAL vs. DOX.*

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.4 mRNA alterations during SAL or DOX acute treatment: role of metabolic substrate and inhibitors

In the next step of this work, we selected a few proteins that were altered by DOX treatment and measured mRNA so that we could confirm whether the differences obtained were accompanied by altered transcripts or instead resulted from other independent mechanisms. Transcripts for ANT, LDH and HIF- 1α were evaluated in hearts acutely treated and perfused with the different perfusates.

4.1.4.1 mRNA content in NP acute hearts

Interestingly, in the non-perfused hearts, a significant difference was found in the ANT transcript, which was two thirds decreased in DOX-treated animals (Fig 26). Lactate dehydrogenase (LDH) and HIF- 1α transcripts were not altered (Fig 26).

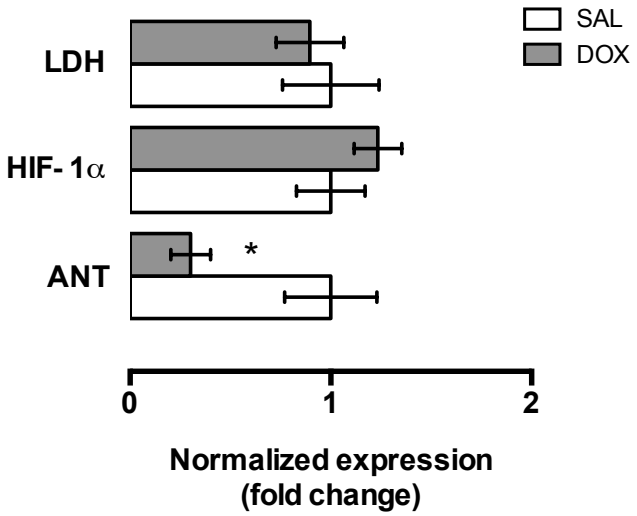

Figure 26. *mRNA content on non-perfused hearts from the SAL and DOX groups. mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Student´s t-test, data are mean ± SEM (n=3-4), * p < 0.05 SAL vs. DOX.*
Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase.*

4.1.4.2 Transcripts in SAL and DOX hearts perfused with glucose: alterations with inhibitors

Hearts from the SAL and DOX group perfused with glucose (TC) or perfused with glucose plus inhibitors were tested for ANT, HIF- 1α and LDH content (Fig. 27). Regarding the ANT transcript, a significant increase was observed in DOX-treated hearts without inhibitors, while the difference was blurred in the presence of the three inhibitor. For the HIF- 1α transcript, TC hearts did not show any difference

between treatments, while in the presence of iodoacetate there was an increase for DOX-treated group compared with SAL, meanwhile DOX hearts with iodoacetate had increased HIF- 1α transcript when compared with DOX hearts from the TC group. LDH transcript did not show any difference during perfusion in any of the conditions studied.

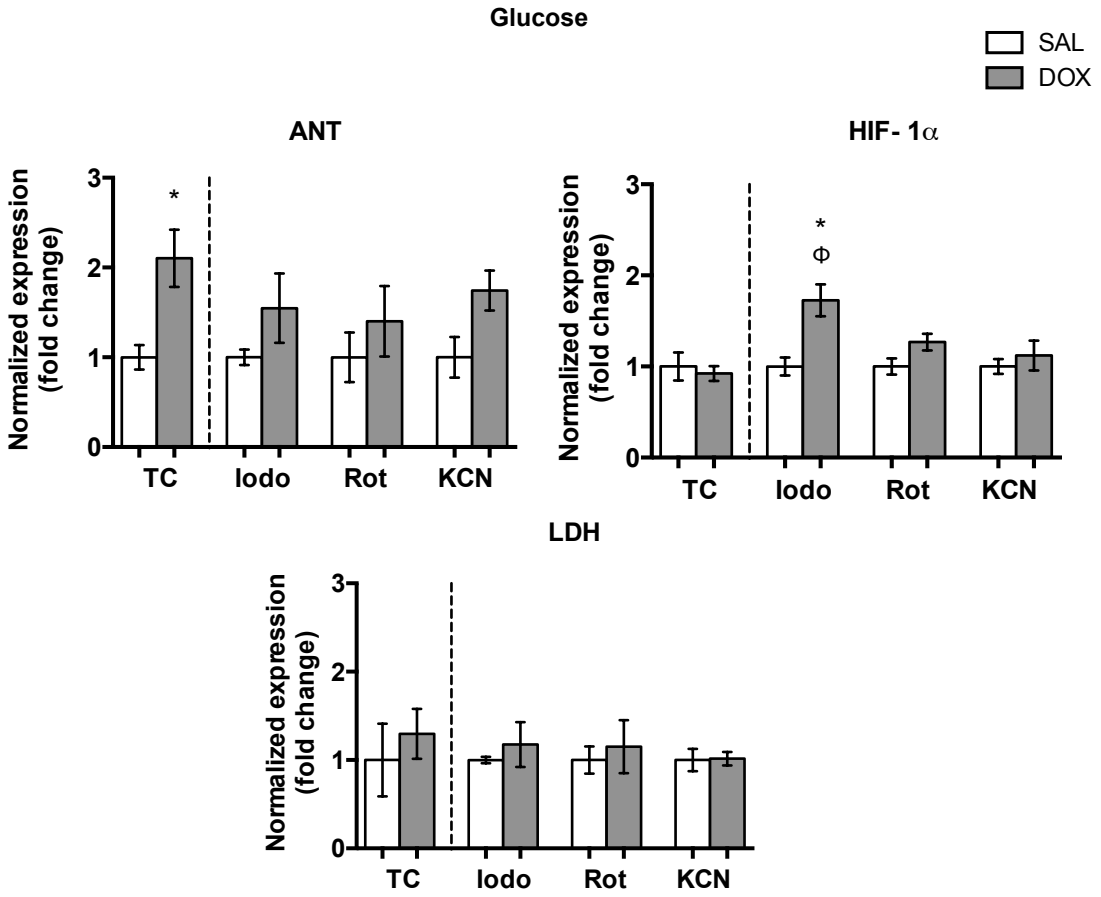

Figure 27. *mRNA content in hearts from SAL or DOX groups and perfused with glucose (TC) or glucose with inhibitors.*

*mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of DOX heart TC group vs. DOX heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=3-4), Φ p < 0.05 TC DOX vs. inhibitor DOX, * p < 0.05 SAL vs. DOX.*

Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase. TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.4.3 Transcripts in SAL or DOX hearts perfused with galactose plus glutamine: alterations with inhibitors

Hearts from the SAL or DOX perfused with galactose plus glutamine (TC) followed by inhibitor titration did not show any differences regarding the three transcripts analyzed, with the exception of the already mentioned lower content in the ANT transcript in TC hearts from the DOX group (Fig. 28). Comparing DOX hearts from the TC condition with DOX hearts perfused with the inhibitors, only the ANT transcript shows an increase on DOX hearts perfused with KCN (Fig. 28). For other transcript or inhibitors no other alterations were found.

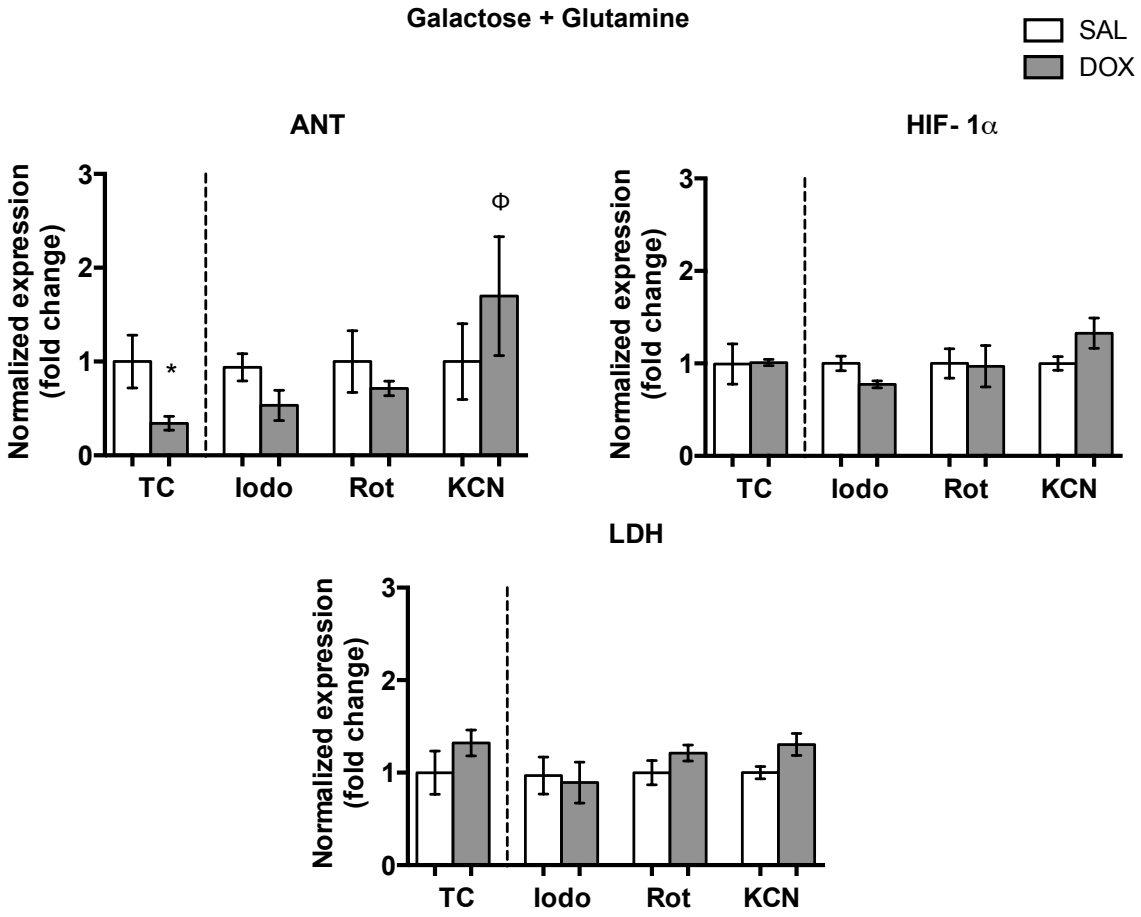

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Figure 28. *mRNA content on hearts acutely treated and perfused with galactose plus glutamine (TC) and galactose plus glutamine with inhibitors.*
mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of DOX heart TC group vs. DOX heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=3-4), Φ p < 0.05 TC DOX vs. inhibitor DOX.
Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase. TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.4.4 Transcripts in SAL or DOX hearts perfused with octanoate plus malate: alterations with inhibitors

By using the octanoate plus malate perfusion protocol without (TC) and with inhibitors, we only detected alterations on the LDH transcript when the hearts were titrated with KCN, with an increase of mRNA measured in DOX hearts comparing with SAL hearts. Also LDH and ANT transcripts in DOX hearts titrated with KCN shows an increase compared with TC group.

No other differences related with treatment (SAL vs. DOX) or perfusion protocol (TC vs. inhibitor) were observed (Fig. 29).

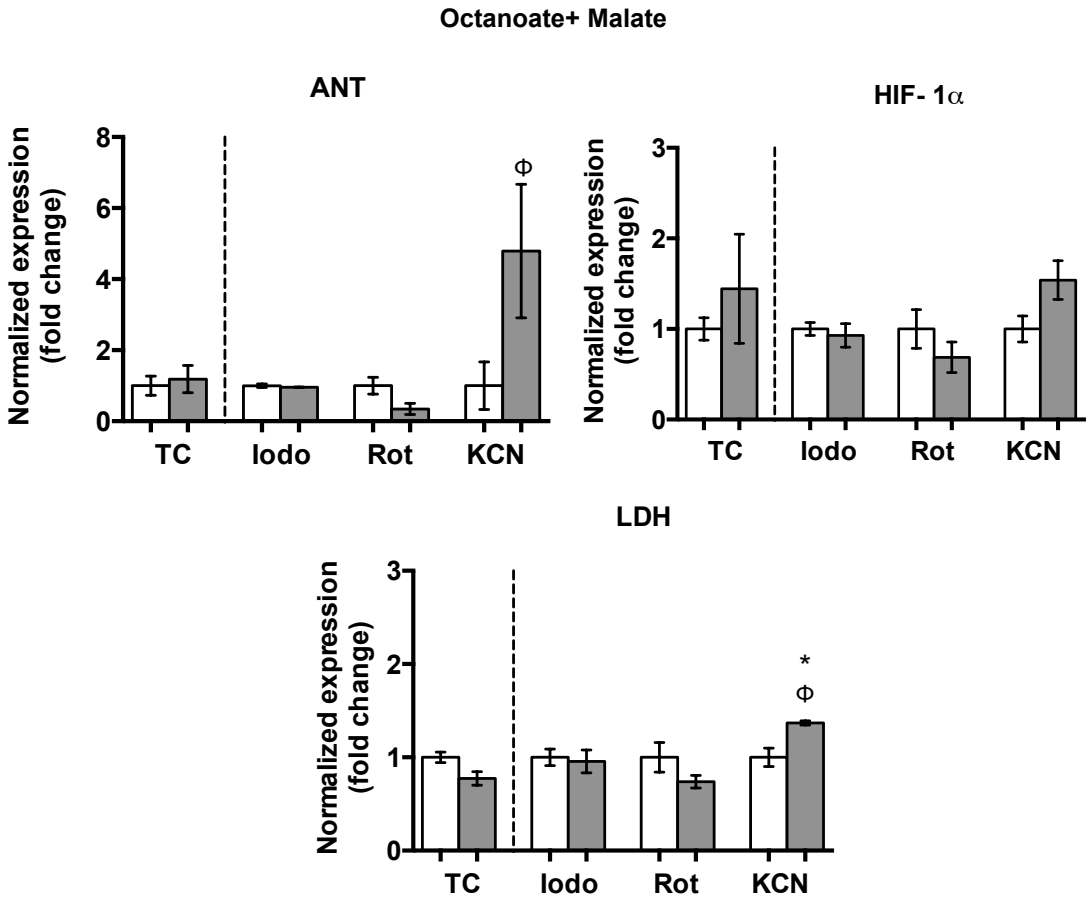

Figure 29. *mRNA content on hearts acutely treated and perfused with octanoate plus malate (TC) and octanoate plus malate with inhibitors.*

*mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of DOX heart TC group vs. DOX heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=3-4), Φ p < 0.05 TC DOX vs. inhibitor DOX, * p < 0.05 SAL vs. DOX.*

Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase. TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.1.5 Discussion of acute treatment results

Metabolic regulation is intimately linked with cardiac function. This metabolism-function relationship is important in several conditions, such as during DOX treatment, resulting in to cardiac hypertrophy and heart failure, a drop of energy and consequent contractile dysfunctions [245].

Heart failure results in shortness of breath and peripheral edema in patients [245], which is associated with an enlarged ventricular chamber and increased heart mass [264]. Under normal conditions, the majority ATP formation in the heart originates from mitochondrial oxidative phosphorylation, with the remainder derived from glycolysis and GTP formation in the TCA cycle [265]. However, during heart failure, the heart suffers from a depletion of ATP due to decreased ability to generate ATP by oxidative metabolism, and thus is unable to effectively transfer the chemical energy from the metabolism of carbon fuels to contractile work [266,267]

Mitochondrial dysfunction as evidenced by decreased mitochondrial respiration and reduced ATP production is consistently observed when systolic dysfunction occurs [265,268]. Taking into account that mitochondrial damage is described as an early event in the progression to heart failure, the alteration in mitochondrial respiratory capacity may trigger unknown compensatory mechanisms that can sustain energy production at multiple levels during the stage of compensated hypertrophy, although cardiac dysfunction is not available. It is expected that the same or similar type of cardiac remodeling may be occurring during DOX administration. To explore the different faces of DOX-induced metabolic remodeling, we used one acute and one sub-chronic treatment protocol. The acute protocol would mimic the immediate

consequences of a bout of treatment course on heart metabolism. We used three distinct metabolic fuels and three inhibitors to try to pinpoint alterations in cardiac metabolism that otherwise would remain silent under basal conditions.

In the present study, we observed that DOX acute treatment influences heart weight, causing a decrease in heart mass. This result is in agreement with several other acute treatment studies that have demonstrated that DOX decrease heart mass [58,88,269]. Although the mechanism is hard to understand, especially because one single treatment was used, it may be that some loss of cells may be occurring through apoptosis [270], although this would probably compromise cardiac function, which was not observed at the basal level.

Taking into account that cardiac metabolism regulates contractibility [245], the author's objective was to force metabolic changes in the heart in order to detect whether DOX would alter cardiac metabolic performance. Previous evidences suggest that the contractile performance is greater when the heart is oxidizing more glucose and lactate in alternative to fatty acids [271]. One general observation from this part of the work is that under TC conditions, SAL and DOX hearts have similar function, as measured by the heart rate and RPP, except when hearts were perfused with glucose, where a higher heart rate was observed in DOX hearts. Hence, by analyzing the results in the acute treatment protocol, where SAL and DOX hearts were submitted to perfusion with different substrates and inhibitors, perfusion with a glucose buffer was optimal for the promotion of glycolysis in the hearts. However, with the addition of iodoacetate on the perfusate, and with an expected GAPDH inhibition, we observed an impairment of both SAL and DOX heart rate and RPP at

higher concentrations, suggesting that iodoacetate inhibits the glycolytic pathway, independently of the SAL or DOX treatment. In the presence of rotenone or KCN, both SAL and DOX hearts suffered a small decline of heart rate and RPP, without differences between treatments, suggesting that after an acute treatment there are still no differences in mitochondrial function that can be detected by this protocol. The use of galactose with glutamine in the perfusion buffer allowed us to increase the TCA cycle and OXPHOS activities, decreasing the cardiac glycolytic flux. It is known that in the absence of glucose, galactose cannot be metabolized and follow the glycolytic pathway [239]. Hence, we would expect that the majority of energy would be produced by OXPHOS [242]. During perfusion, the presence of iodoacetate induced a decline of RPP on SAL hearts, with significant differences between treatments at the highest concentrations, demonstrating that DOX hearts are not affected by the inhibitor, as opposed to SAL hearts. The same effect appears with the use of KCN, with SAL hearts showing more susceptibility comparing with the DOX hearts. The effect of iodoacetate, a glycolytic inhibitor, on cardiac performance sustained by the galactose plus glutamine buffer is very surprising, since we would expect minor effects. Nevertheless, iodoacetate is an irreversible inhibitor of cysteine peptidases, which may not exclude a selective effect, including removal of free GSH [272], which may lead to cardiac failure [273,274]. Regarding the fact that under stressful conditions, the heart favors glycolysis as the principal energy source [245], it is assumed that DOX acute treatment led to fast metabolic adaptations, which may be beneficial for hemodynamic response in the short term. This may also explain the higher heart rate in TC experiments. Moreover, the use of galactose plus glutamine forces hearts to potentiate the TCA cycle on DOX hearts, which may have resulted in

an improvement of hemodynamic responses, proven that OXPHOS is not affected. The presence of rotenone did not show significant differences between SAL and DOX hearts, however RPP data shows that DOX had a higher value than SAL hearts, confirming an initial positive advantage. Perfusion results with octanoate plus malate with addition of iodoacetate or rotenone were similar, with an equivalent decreased in heart rate and RPP in both SAL and DOX hearts. The addition of KCN also decreased both hemodynamic parameters, with significant differences in the heart rate of SAL hearts comparing with DOX hearts, although the RPP was not affected. Although fatty acid β-oxidation pathway provides a major energy source [275], previous results suggest that DOX inhibits fatty acid oxidation in the heart [186,276]. In our case, the results from the acute study show that cardiac performance with these substrate was similar for both groups. It is not expected that under this protocol for acute treatment, major drug-related alterations in β-oxidation occurred. Also our results show that octanoate plus malate were not able to sustain cardiac contractility when hearts were perfused with the different inhibitors, showing the relevance of the interplay between different metabolic pathways.

The rates of flux through the various metabolic pathways are controlled by both the degree of expression of key metabolic proteins and complex pathway regulation that is exerted by both allosteric regulation of enzymes and substrate/product relationships [265]. Trying to understand the perfusion results, selected proteins related with glycolysis and mitochondria of SAL and DOX hearts were analyzed. In non-perfused hearts, p62 and Beclin-1 were increased in DOX hearts, demonstrating that autophagic protein degradation was impaired by DOX due to the accumulation of these proteins in cell. However, contradictory reports are found in the literature,

showing that for high DOX concentrations, autophagy is blocked [270] while others suggest that autophagy is activated by DOX in order to protect the cell from damage [277]. Proteins related with mitochondrial biogenesis, such as TFAM and PGC-1α showed a decrease in DOX hearts, suggesting an impairment of mitochondrial biogenesis, which was also described before [278,279]. This fact alone can suggest that hearts may fail when subjected to a prolonged stress that triggers an increase in mitochondrial biogenesis to compensate an increased overload. This can occur during physical exercise or pregnancy [280].

Stability of cellular proteins is fundamental for the regulation of a variety of cell functions, such as growth, differentiation or even cell death [281]. Taking into account that half-life of different proteins can vary between a few minutes and days [281,282], the content of proteins from hearts perfused for over 55 minutes would be different (without or with inhibitor) from the content in non-perfused hearts.

In hearts perfused with glucose and different inhibitors, an increased protein amount between treatments was observed for p62, when iodoacetate or rotenone were used as inhibitors, and on Hsp90 when rotenone was present, suggesting that autophagy was impaired under those conditions. Both proteins are related with protein degradation, by assisting proteins to fold properly or tag proteins to follow degradation. In fact, accumulations of tagged proteins indicate that cellular accumulates in the cardiomyocyte, causing downstream damage and mostly likely increasing oxidative stress [277]. However, for glycolytic proteins, alterations were not observed comparing SAL versus DOX hearts showing that any alteration at this level was independent of protein content. In hearts perfused with galactose plus

glutamine and inhibitors, proteins involved in the conversion of glucose to glucose-6-phosphate, such as hexokinase I and hexokinase II were decreased in DOX hearts titrated with iodoacetate. These alterations could be justified by the down-regulation of glycolytic rates, induced by the presence of iodoacetate [283]. However, this result is not correlated to the hemodynamic rates described previously.

Proteins related with autophagy, such as Beclin-1 and p62 were also increased in DOX hearts after perfusion with iodoacetate, while Hsp90 and ubiquitin were decreased. These results suggest impairment in the degradation of proteins on DOX hearts compared with SAL hearts. Mitochondrial proteins ANT and TFAM were decreased in DOX hearts perfused with iodoacetate. This interesting result suggests a bioenergetics collapse that causes a decrease of mitochondrial-relevant proteins, which may result from increased mitophagy. A downstream result may be impairment of mitochondria function, since the ANT is involved in the translocation of ATP/ADP on mitochondria [284] while TFAM participates in mitochondrial genome replication [15]. It is also important to notice that a previous report demonstrated that TFAM silencing results in a progressive decline in ETC activities and mitochondrial ATP production [285].

When octanoate plus malate were used in the perfusate, no alterations in glycolytic proteins were observed. Only p62 was increased in DOX hearts when all the three inhibitor were present, supporting that an impair of autophagy is happening in DOX hearts compared with SAL hearts, most likely resulting from bioenergetics collapse, added to an already compromised autophagic flux.

Comparing glycolytic proteins of TC hearts with hearts perfused with glucose and inhibitors, differences were detected in both SAL and DOX hearts, showing a decrease on Hexokinase II, GAPDH and LDHA proteins for all the three inhibitors used. This suggests that the presence of the inhibitors may again lead to a bioenergetics collapse and possible degradation/decrease expression of metabolic enzymes. Interestingly, it was previously demonstrated that a decrease on mitochondrial activity may also result in a lower association of glycolytic enzymes with mitochondrial membranes [286]. However, by measuring PKM 1/2 and PDH in hearts perfused with glucose with iodoacetate, both SAL and DOX hearts show an increase comparing with respective TC hearts, this suggest that possibly an adaptation to counteract the inhibitory effects of iodoacetate occurred in order to increase efficiency of conversion of pyruvate to acetyl-CoA. When measuring other proteins in hearts perfused with glucose in the absence or presence of inhibitors, proteins related with mitochondria function and possible quality control, such as PGC 1α, TFAM, Hsp90 and p62 showed that for both SAL and DOX groups these proteins were decreased in comparison with TC hearts, suggesting that the addition of inhibitors does cause alteration in cell proteins, specially those related with mitochondrial function, as previously documented [287,288]. Similar results were described with hearts perfused with galactose plus glutamine, whereas hearts perfused with inhibitors showed decreased amount of glycolytic proteins such as hexokinase II, PKM 1/2 and LDHA, again confirming that a possible bioenergetic collapse causes overall down-regulation of multiples proteins. For hearts titrated with iodoacetate, an increase in GAPDH was detected. This is a very interesting effect suggesting that the hearts are trying to overcome the inhibition causes in the

glycolytic enzyme by iodoacetate. Again and similar to hearts perfused with glucose, hearts perfused with galactose plus glutamine showed a decline on ANT, CypD, PGC 1α, TFAM, Hsp90 and p62 protein, equivalent in both SAL and DOX hearts when comparing with respective TC hearts, again confirming the general (with exceptions) down-regulating effect of the inhibitors on protein content [287,288]. On hearts perfused with octanoate plus malate, hexokinase I and hexokinase II, as well as LDHA showed a decrease for all three inhibitors, again confirming the trend that glycolytic proteins are decreased when hearts suffer bioenergetics collapse [286].

Considering the protein results previously described, we next evaluated mRNA for protein that influence glycolysis, such as HIF- 1 α and LDH [289], and also the ANT. On non-perfused hearts, only ANT showed decrease of mRNA content on DOX hearts, consistent with previous reports [290]. Meanwhile, when glucose perfused hearts were studied, DOX TC hearts showed an opposite result for ANT, with an increase of ANT in hearts DOX-treated, more studies should be done to take additional conclusions about this result, although the perfusion time may have caused a shift on the trend of mRNA level difference. An increase was observed for HIF- 1 α in hearts perfused with glucose in the presence of iodoacetate, supporting the idea that DOX hearts promote adaptations in glycolysis. By analyzing hearts perfused with galactose plus glutamine, differences were detected in TC hearts in ANT mRNA, with a decrease of mRNA contents in DOX, similar to non-perfusion hearts. Hearts perfused with octanoate plus malate showed a difference between treatments on transcript LDH in the presence of KCN showing that KCN promote susceptibility in DOX hearts inducing toxicity.

The results from this section, although not allowing for a conclusive explanation of the differences found, appear to confirm that our experimental protocol is effective in pinpointing metabolic defects under drug-induced disease or even the pathophysiology of cardiovascular diseases.

As general comment, it appears that after an acute treatment, DOX hearts suffer a metabolic remodeling, which, if something, allows them to be better prepared for metabolic inhibition. It is also clear that proteins related with mitochondrial biogenesis are decreased, which may have long-term effects in the heart. Finally, autophagy is apparently compromised in DOX-treated hearts, which raises some doubts on the potential protective role of that quality control mechanism during an acute DOX treatment.

4.2. Doxorubicin Treatment – Sub-chronic protocol

Doxorubicin cardiotoxicity can be cumulative and dose-dependent, and most symptoms appear after the completion of treatment. To minimize these symptoms lower doses of DOX are administrated during an extensive period [291].

We next used a sub-chronic treatment protocol in Wistar-Han rats, adapted from the original treatment schedule introduced by Wallace *et al* [292] and used in our laboratory [88]. Using this treatment protocol (7+1 weeks), the same experimental strategy as described for the acute protocol was followed regarding time control titration with inhibitors and protein and mRNA analyses.

4.2.1 Doxorubicin sub-chronic treatment endorse a decrease on Wistar rat body weights

Animals from both SAL (n=40) and DOX (n=38) were weighted during the eight weeks of the treatment course. The last measurement was right before the sacrifice.. During the sub-chronic treatment, a slower body weight gain was observed in the DOX-treated animals that became significantly different starting from the 3rd week of DOX treatment comparing with the SAL group (Fig. 30).

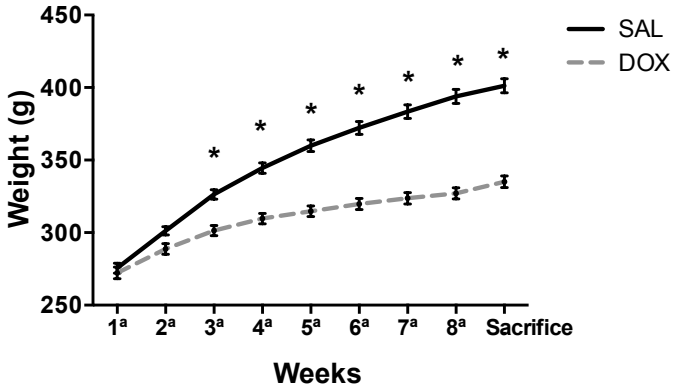

Figure 30. *Body weight of saline (SAL) and doxorubicin (DOX) sub-chronically treated animals. Student´s t-test, data are mean ± SEM, SAL n= 40, DOX n= 38* p < 0.05 SAL vs. DOX*

After animal sacrifice of the animals hearts were harvested and weighted. No differences between treatments regarding heart weight (Fig. 31B) were found, the ratio heart although over body mass shows as a increase in the DOX group, due to the smaller body weight of animal in sacrifice (Fig. 31A).

As during the acute study, hearts were observed during extraction and none of them showed any size, color or morphological alterations.

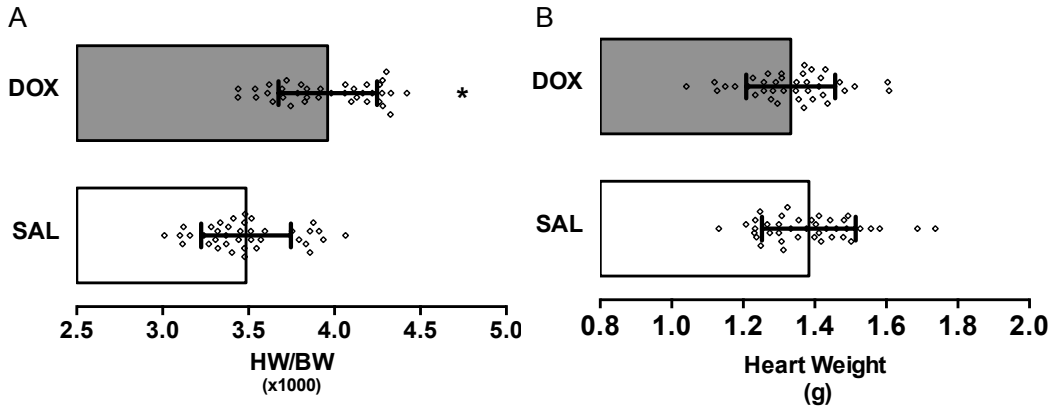

Figure 31. *Heart weight / body weight ratio (A) and heart weight (B) of saline (SAL) and doxorubicin (DOX) sub-chronically treated animals. Student´s t-test, data are mean ± SEM, SAL n= 40, DOX n= 38. * p < 0.05 SAL vs. DOX*

4.2.2 Sub-chronic DOX-heart perfused show hemodynamic rates disturbance under metabolic inhibition- role of substrate

In this protocol, and has described before, glucose and galactose plus glutamine were used as metabolic supply for the heart and the three inhibitors were added separately. The use of octanoate plus malate as substrate was not used in the sub-chronic treatment, due to the lack significant results on acute treatment, and further financial and short time difficulties.

4.2.2.1 Time Control (TC) hearts under sub-chronic treatment

Saline and DOX hearts were perfused with two metabolic fuels (glucose and galactose plus glutamine) separately, during the total time of heart perfusion with inhibitors. These time controls hearts served to verify whether hearts can withstand the total perfusion time. No differences between SAL and DOX hearts, or even

during the total perfusion time were found (Fig. 32A and B). With galactose plus glutamine buffer, both TC hearts showed a significant difference between treatments in RPP parameters at 55 min of perfusion, with no significant consequence on heart rate (Fig. 32C). Nevertheless, and despite the fact that the effect is not statistically significant, a trends towards a depressed function with perfusion time was found.

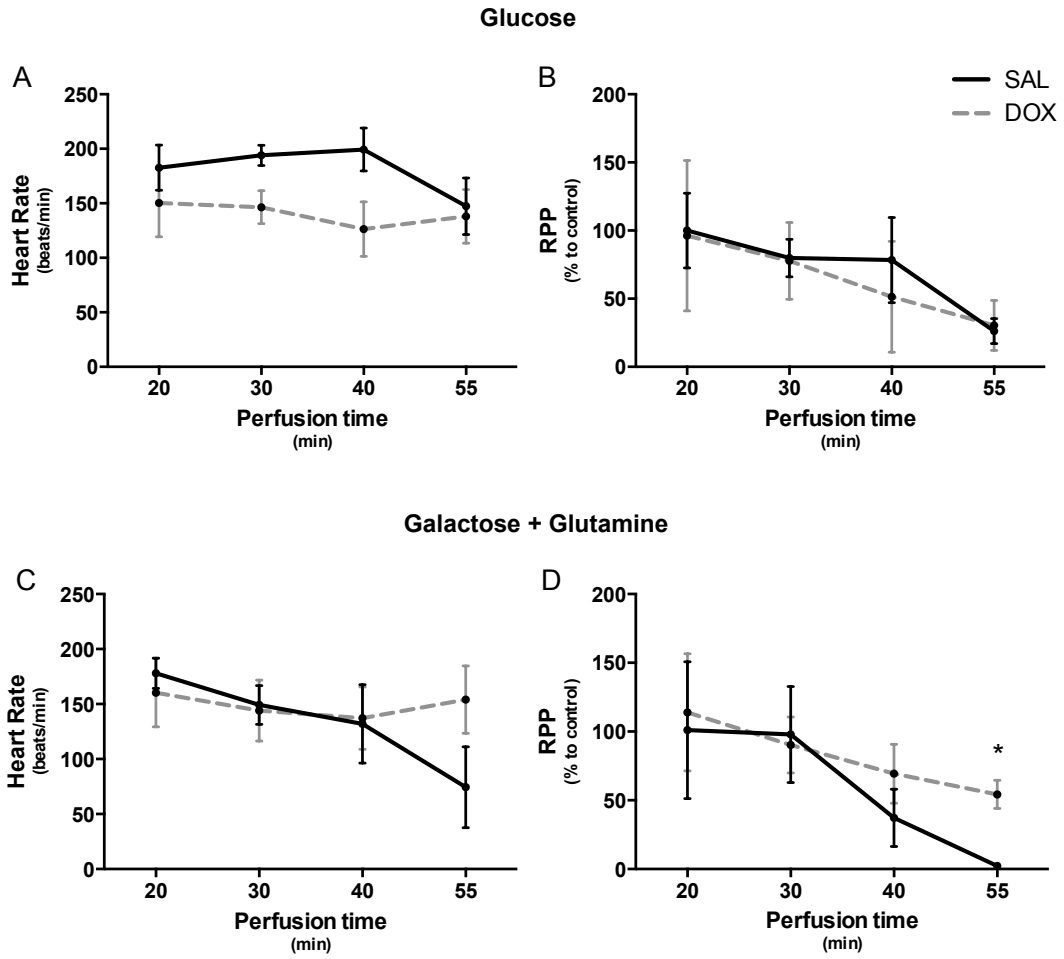

Figure 32. *Time control perfusion of SAL or DOX hearts sub-chronically treated. Perfusion with glucose (A and B, n=5-6) and galactose plus glutamine (C and D, n=5-6).*
*Comparisons between SAL and DOX groups for the same perfusion time were performed using a Student´s t-test. Comparisons of perfusion time vs. initial perfusion (indicated as 20) were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, * $p < 0.05$ SAL vs. DOX.*

4.2.2.2 Effect of sub-chronic DOX treatment on heart hemodynamic behavior under metabolic inhibition: glucose as the fuel source

The heart rate and RPP from hearts perfused with glucose, followed by the addiction of the three inhibitors was found (Fig. 33). The use of iodoacetate decreased the hemodynamic parameters analyzed, however the decline is only significant for the SAL group in the final recovery phase, comparing with the initial perfusion, in terms of heart rate (Fig. 33A) and RPP (Fig. 33B). When rotenone was used as inhibitor (Fig. 33 C and D) no SAL vs. DOX differences were observed. When KCN was used during the perfusion, both SAL or DOX groups shows a decrease at 0.25, 0.5, 0.75 and 1mM KCN, when compared with initial glucose perfusion (0) in the heart rate (Fig. 33E), with the significant difference maintained under the final glucose perfusion. Regarding the RPP parameter (Fig. 33F), only DOX hearts were affected by the concentrations of 0.25, 0.5, 0.75 and 1mM KCN, leading to an impairment in the recovery phase.

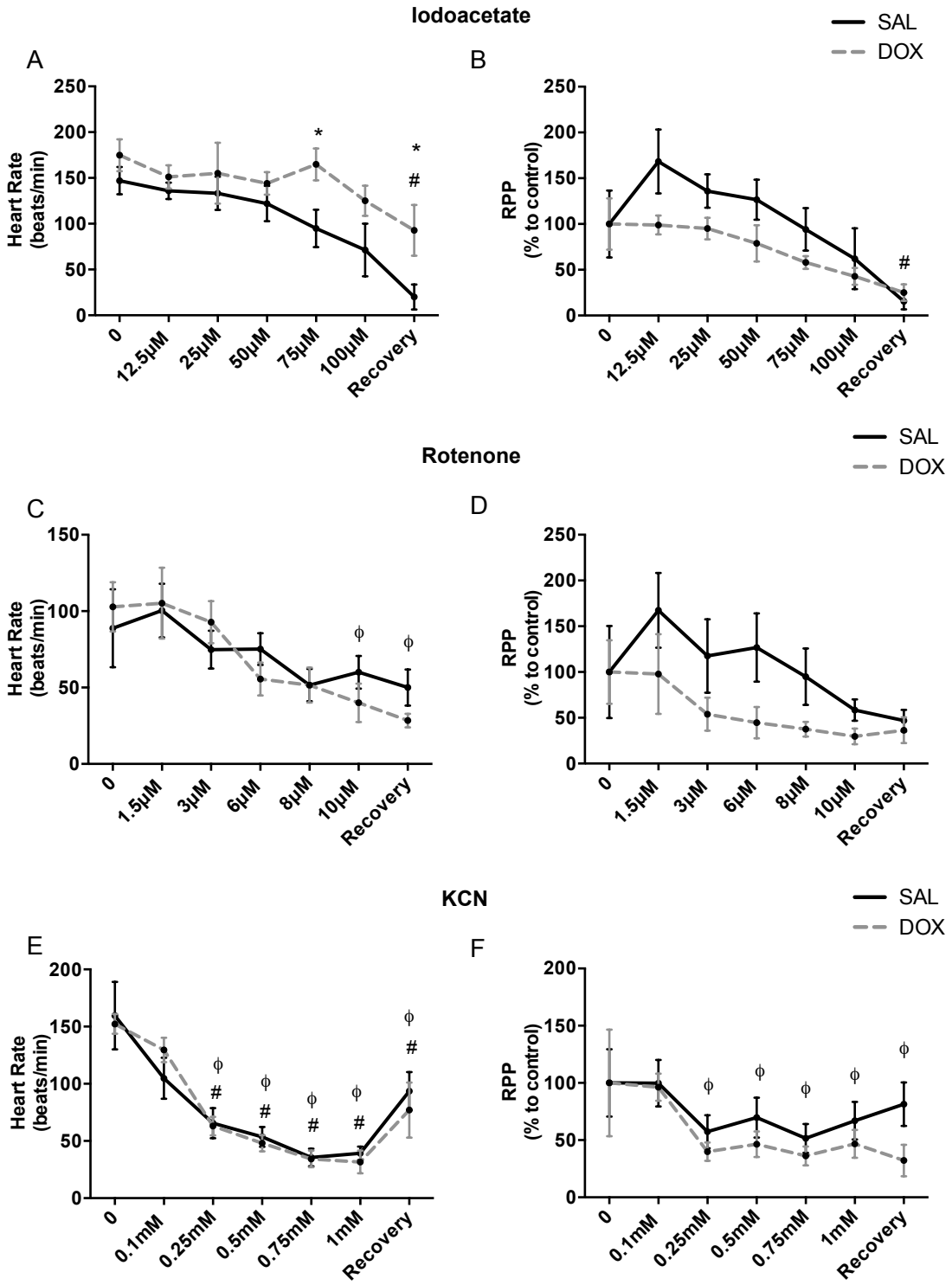

Figure 33. *Heart perfusion with glucose and titration of iodoacetate (A and B, n=4-6), rotenone (C and D, n=5-6) and KCN (E and F, n=5-6).*

*Initial perfusion is represented in the graph as "0", and recovery phase in the absence of inhibitors as "Recovery". Comparisons between SAL and DOX groups for the same perfusion condition were performed using a Student´s t-test. Comparisons of inhibitor concentration vs. initial perfusion (indicated as 0) were performed with one-way ANOVA, followed by the Bonferroni post-test. # $p < 0.05$ SAL vs. 0 , Φ $p < 0.05$ DOX vs. 0, * $p < 0.05$ SAL vs. DOX, data are mean ± SEM.*

4.2.2.3 Effect of sub-chronic DOX treatment on heart hemodynamic behavior under metabolic inhibition: galactose plus glutamine as the fuel source

Using iodoacetate, the results showed that only DOX hearts had decreased heart rates during the perfusion with 50, 75 and 100 μM of iodoacetate (Fig. 34A), while the SAL heart maintained the same values. In the recovery phase using galactose plus glutamine, DOX hearts were not able to recover, showing a significant difference between SAL and DOX hearts. Meanwhile, the same inhibitors caused a decline in the RPP value for both groups (Fig. 34B) with 50, 75 and 100 μM iodoacetate, plus during the recovery phase, when comparing with the initial perfusion (Fig. 34).

The use of rotenone only affected heart rate in the SAL group (Fig. 34C) for the concentration of 1, 1.5 and 2 μM, with hearts from both groups showing a significant reduction in the recovery phase. No differences were detected in RPP when rotenone was used (Fig. 34D). By using KCN as inhibitor (Fig. 34E), the heart rate showed a decline in SAL group for 0.2, 0.3 and 0.5 mM KCN, while for DOX hearts only 0.3 and 0.5 mM of KCN showed a significant decrease. Both groups had the ability to recover post-inhibitor. Figure 34F shows that the SAL group had decreased RPP when KCN was used, while DOX initiated only the decline for 0.1 mM KCN, with hearts unable to recover.

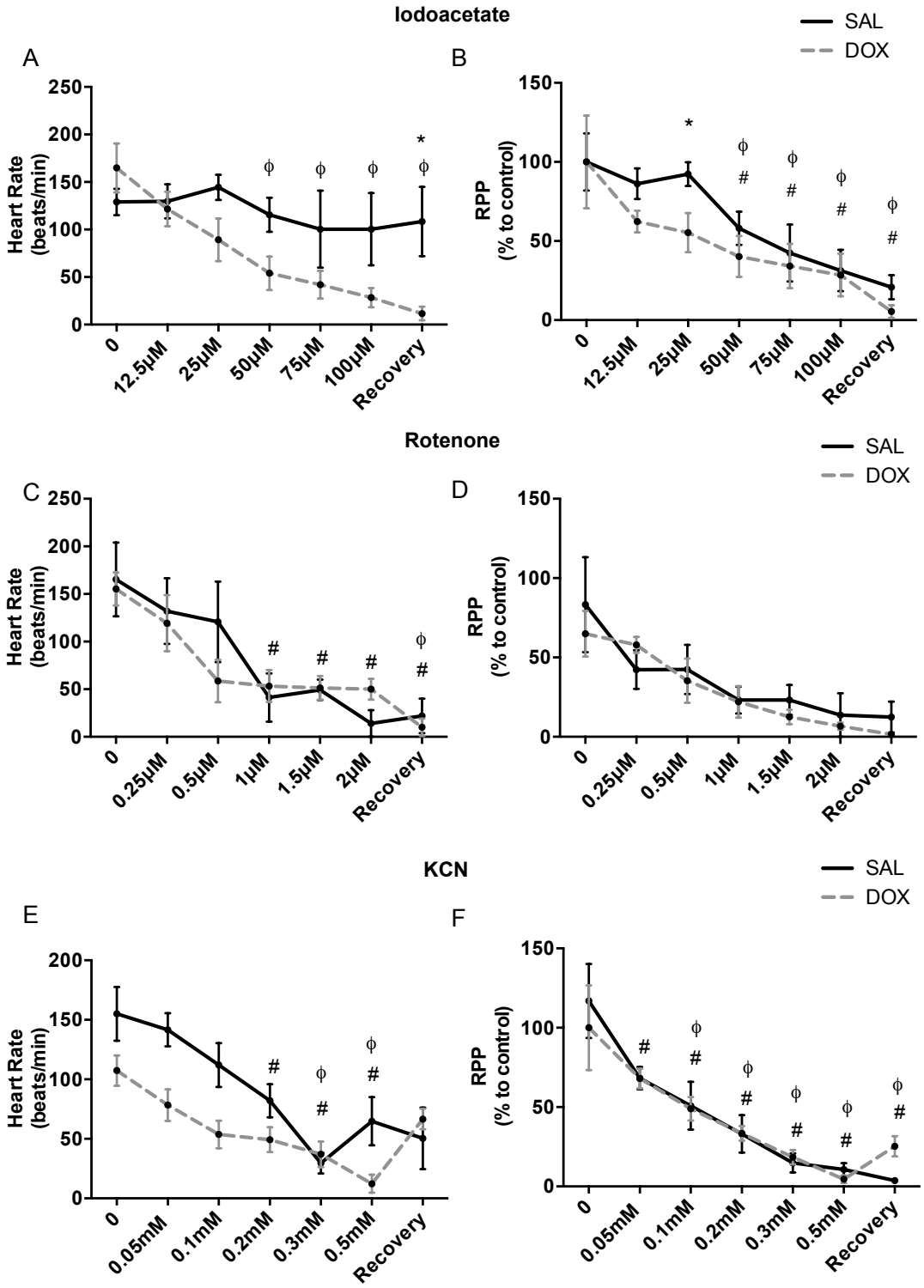

Figure 34. *Heart perfusion with galactose plus glutamine and titration of iodoacetate (A and B, n=5-6), rotenone (C and D, n=5-6) and KCN (E and F, n=4-6).*

*Initial perfusion is represented in the graph as "0", and recovery phase in the absence of inhibitors as "Recovery". Comparisons between SAL and DOX groups for the same perfusion condition were performed using a Student´s t-test. Comparisons of inhibitor concentration vs. initial perfusion (indicated as 0) were performed with one-way ANOVA, followed by the Bonferroni post-test. # $p < 0.05$ SAL vs. 0 , Φ $p < 0.05$ DOX vs. 0, * $p < 0.05$ SAL vs. DOX, data are mean ± SEM.*

4.2.3 Protein alterations in perfused hearts in SAL and DOX sub-chronically treated rats: role of substrates and metabolic inhibition

After the perfusion, SAL and DOX treated hearts as well as the non perfused hearts, were collected and were analyzed by Western blotting to identify alterations resulting from SAL vs. DOX treatment, as well as before vs. after inhibitor titration.

4.2.3.1 Non-Perfused (NP) SAL and DOX hearts after sub-chronic treatment

Similarly to the acute treatment protocol, glycolytic proteins were also analyzed in non-perfused hearts submitted to a sub-chronic treatment with SAL or DOX (Fig. 35). Regarding the different proteins analyzed, only Hexokinase I increased in the DOX heart as compared to the SAL group.

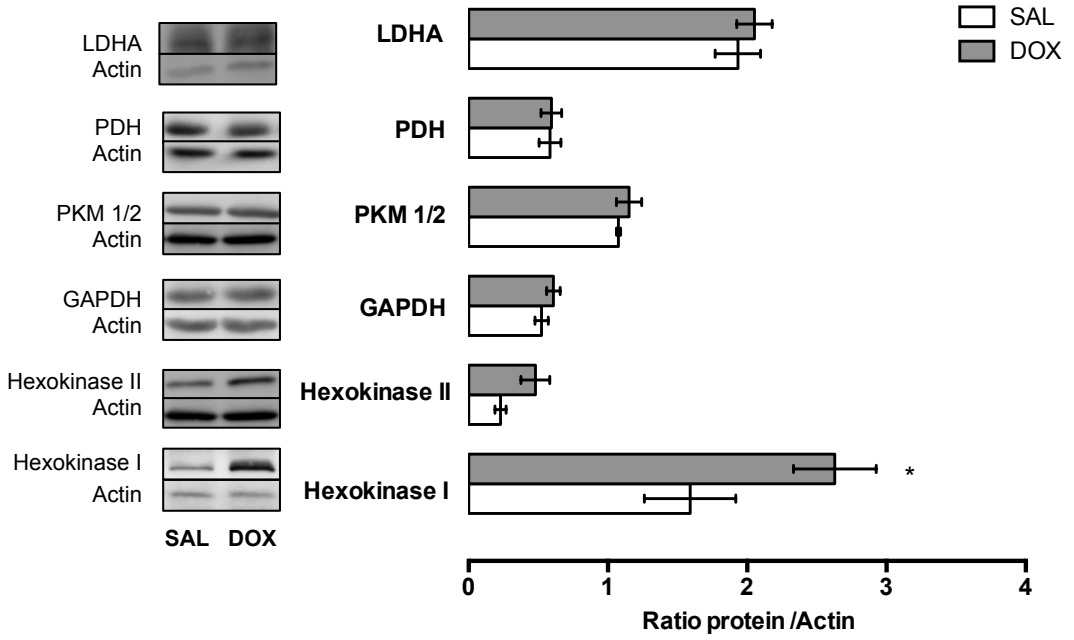

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Figure 35. *Amount of protein related to the glycolytic pathway was measured by Western blotting.*

*Representative Western blot for the mentioned proteins together with their respective loading control. Student´s t-test, data are mean ± SEM, (n=5-6), * p < 0.05 SAL vs. DOX.*

Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa).*

Several proteins were also analyzed in SAL vs. DOX samples from sub-chronically-treated rats. No differences were found between groups.

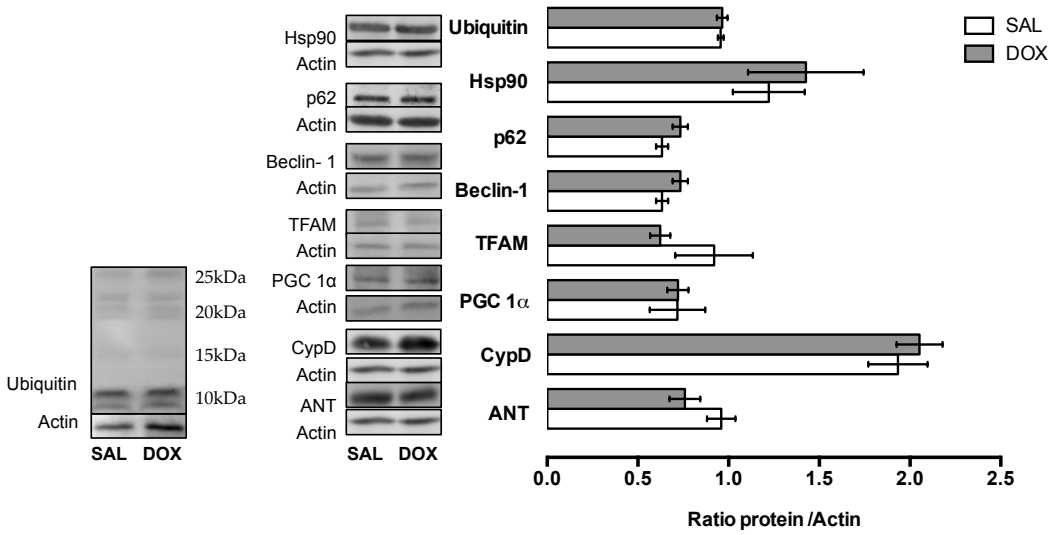

Figure 36*. Protein amount on non-perfused hearts was measured by Western blotting.*

For ubiquitin quantification, a larger area was quantified for each group. Representative Western blot for the mentioned proteins together with their respective loading control. Student´s t-test, data are mean ± SEM, (n=5-6).

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa).*

4.2.3.2 Protein alterations in sub-chronically treated hearts perfused with glucose: role of substrate and metabolic inhibition

Regarding glucose-perfused hearts, when comparing glycolytic proteins in SAL vs. DOX hearts in the presence of the three inhibitors, no differences between treatments were found. However, when differences between TC hearts and inhibitor-treated in the hearts were observed, an increase of Hexokinase II for all the inhibitors was found. This increasing effect also occurred in GAPDH in DOX hearts and PKM 1/2 in both SAL and DOX hearts, although only when iodoacetate was present. For LDHA an increase was observed with iodoacetate and KCN in the DOX hearts when comparing TC, while only for iodoacetate this increased was detected in SAL hearts. Contrarily to the other proteins, the amount of PDH in both SAL and DOX hearts perfused with each of the inhibitors decreased comparing with hearts from TC condition (Fig. 37).

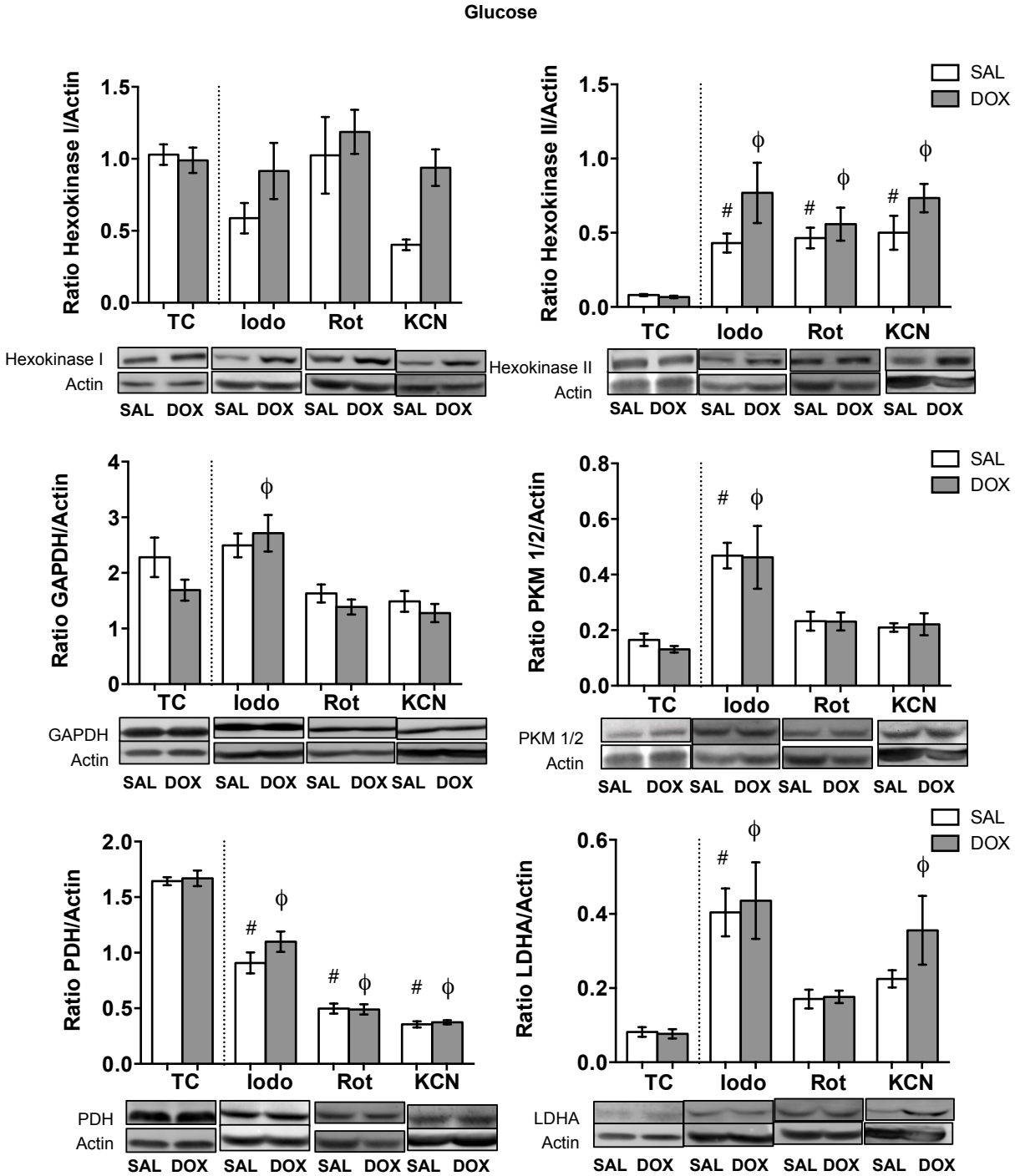

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Figure 37. *Proteins involved in the glycolytic pathway probed in glucose perfused hearts from SAL or DOX-treated rats.*

Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX.

Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

For proteins related with mitochondria and cellular mechanisms of quality control, we detected treatment-related variations, with an increase of p62 and Hsp90 in DOX when compared with SAL hearts (Fig. 38). Regarding comparisons between TC and inhibitor-treated hearts, several proteins shows significant differences, such as CypD, PGC-1α, p62, Hsp90 and ubiquitin, showing a significant decrease when all the inhibitors are present in the perfusate for both SAL and DOX hearts. ANT, TFAM and Beclin-1 proteins showed an increase of protein amount on DOX hearts perfused with iodoacetate comparing with DOX heart from the TC condition. Also SAL hearts perfused with iodoacetate showed an increase of Beclin-1 (Fig. 38).

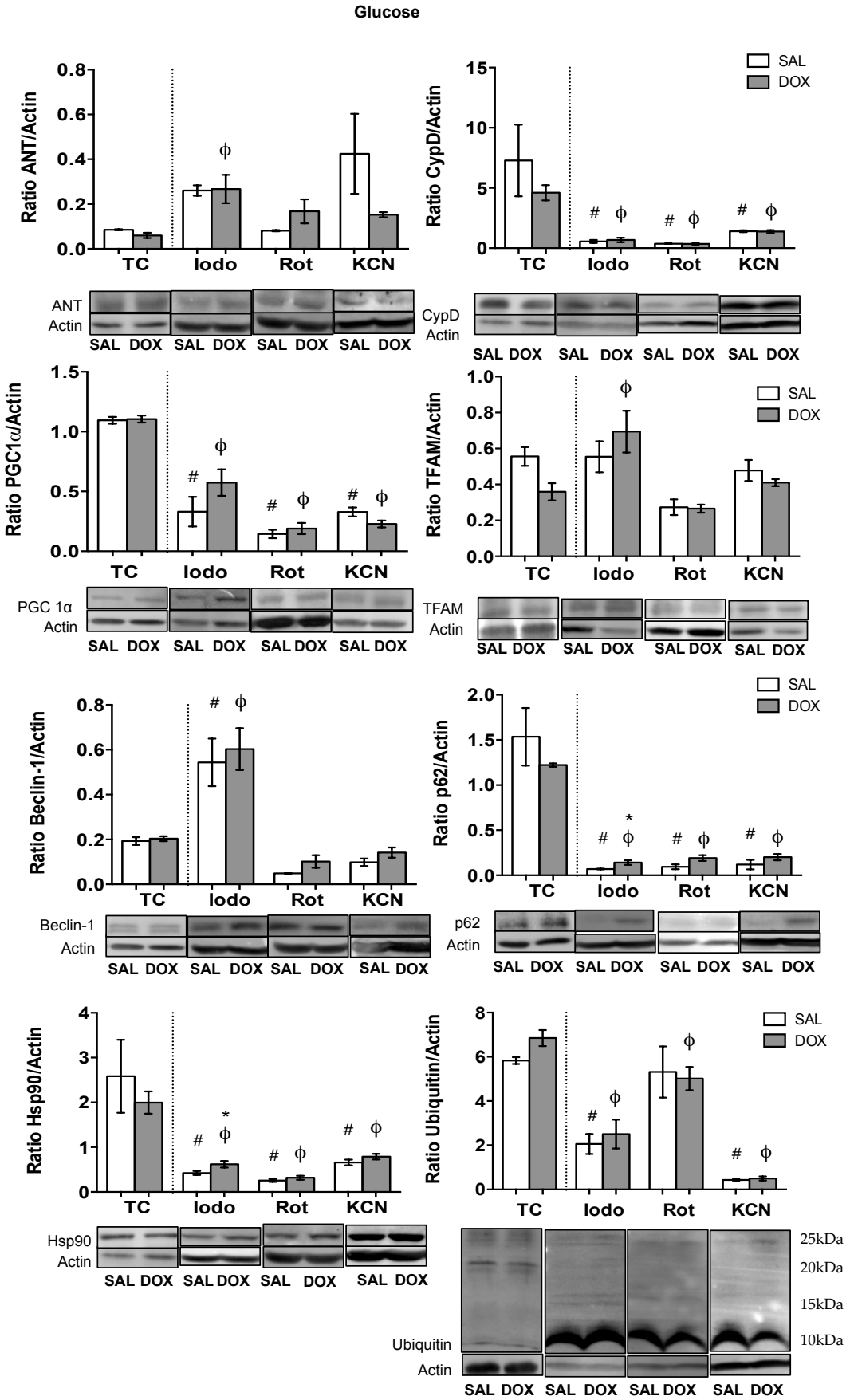

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Figure 38. *Proteins involved in the mitochondria events probed in glucose perfused hearts from SAL or DOX-treated rats.*

*Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. For ubiquitin quantification, a larger area was quantified for each group. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. . Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX, * $p < 0.05$ SAL vs. DOX.*

Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide*

4.2.3.3 Protein alterations with sub-chronically treated hearts perfused with galactose plus glutamine: role of substrate and metabolic inhibition

We performed similar experiments when the hearts were perfused with galactose plus glutamine (Fig. 39). None of the investigated proteins were altered in SAL vs. DOX-treated rats. However, when comparing hearts in TC condition vs. after inhibitor, several differences were found. The use of each of the three inhibitors in the perfusate decreased the amount of Hexokinase II, PKM 1/2 and LDHA in SAL and DOX hearts. A similar decrease with GAPDH occurred in SAL hearts with all inhibitors, while DOX decreased only when the perfusate contained KCN. In the

opposite direction and comparing with hearts in the TC condition, iodoacetate and rotenone increased PDH protein amount DOX hearts, while in SAL hearts the increase occurred only with titration with iodoacetate (Fig. 39).

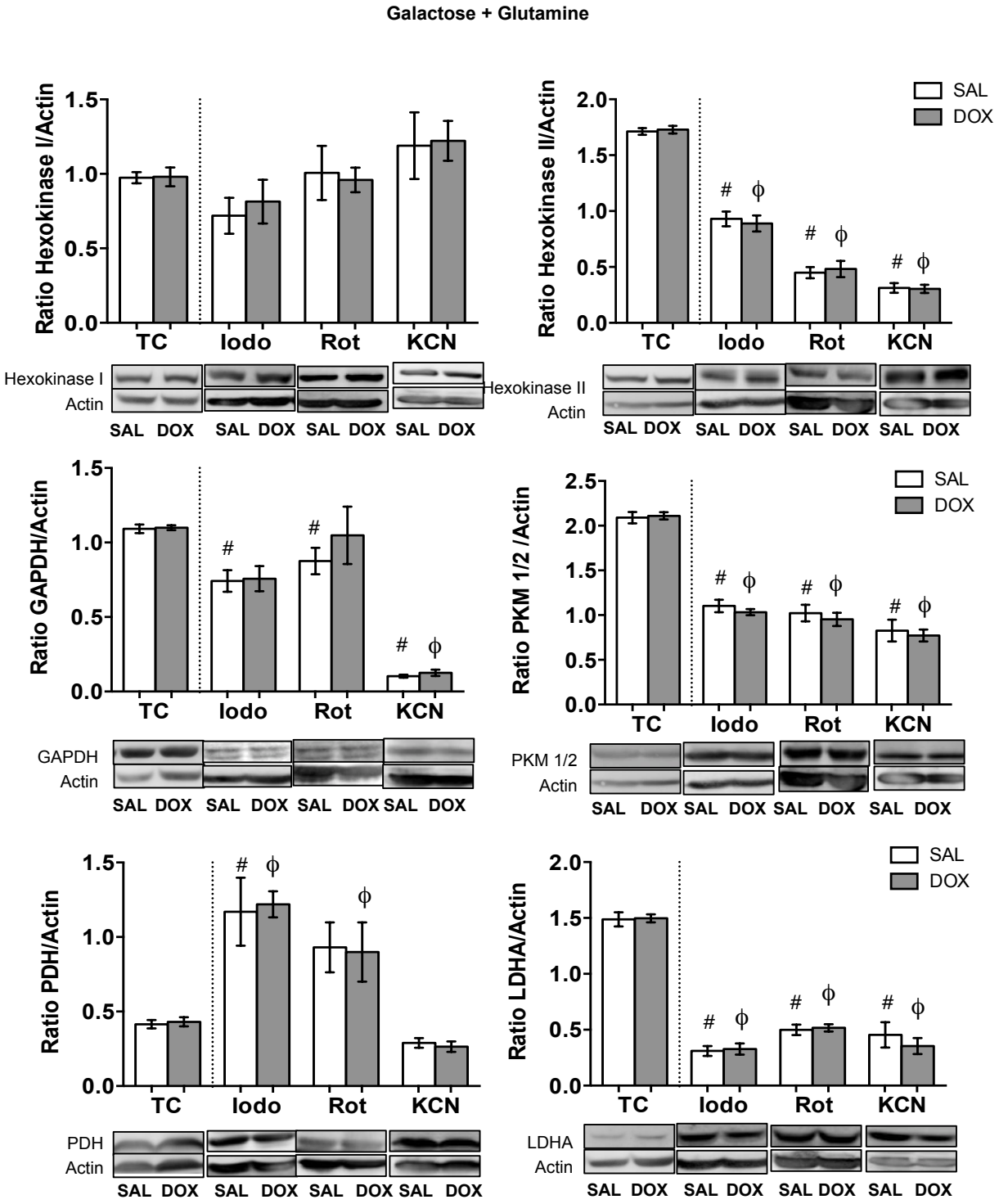

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Figure 39. *Proteins involved in the glycolytic pathway probed in galactose plus glutamine perfused hearts from SAL or DOX-treated rats.*
Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # $p < 0.05$ TC SAL vs. inhibitor SAL, Φ $p < 0.05$ TC DOX vs. inhibitor DOX.
Abbreviations: *Hexokinase I (102kDa); Hexokinase II (102kDa); GAPDH (37kDa)-Glyceraldehyde-3-phosphate dehydrogenase; PKM 1/2 (60kDa)-Pyruvate kinase; PDH (43kDa)-Pyruvate dehydrogenase; LDHA (37kDa)-Lactate dehydrogenase A isoenzyme; Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

p62 also increased in DOX hearts when rotenone was added to the perfusate, while Beclin-1 decreased on DOX hearts in the presence of KCN. By comparing hearts from TC and inhibitor-treated protocols, we observed a decrease on ANT, Beclin-1 and p62 proteins for all inhibitors in DOX hearts, the same effect was observed in SAL hearts with exception of Beclin-1 with KCN. The same decrease was detected also on CypD and PGC-1α when KCN was present in the perfusate for both hearts. Interestingly, CypD showed an increase of protein amount for SAL and DOX hearts perfused with rotenone and a decrease on SAL hearts perfused with iodoacetate. The amount of detected ubiquitin was increased in the presence of all inhibitors in TC vs. inhibitors-treated hearts, with exception of SAL hearts in the presence of iodoacetate (Fig. 40).

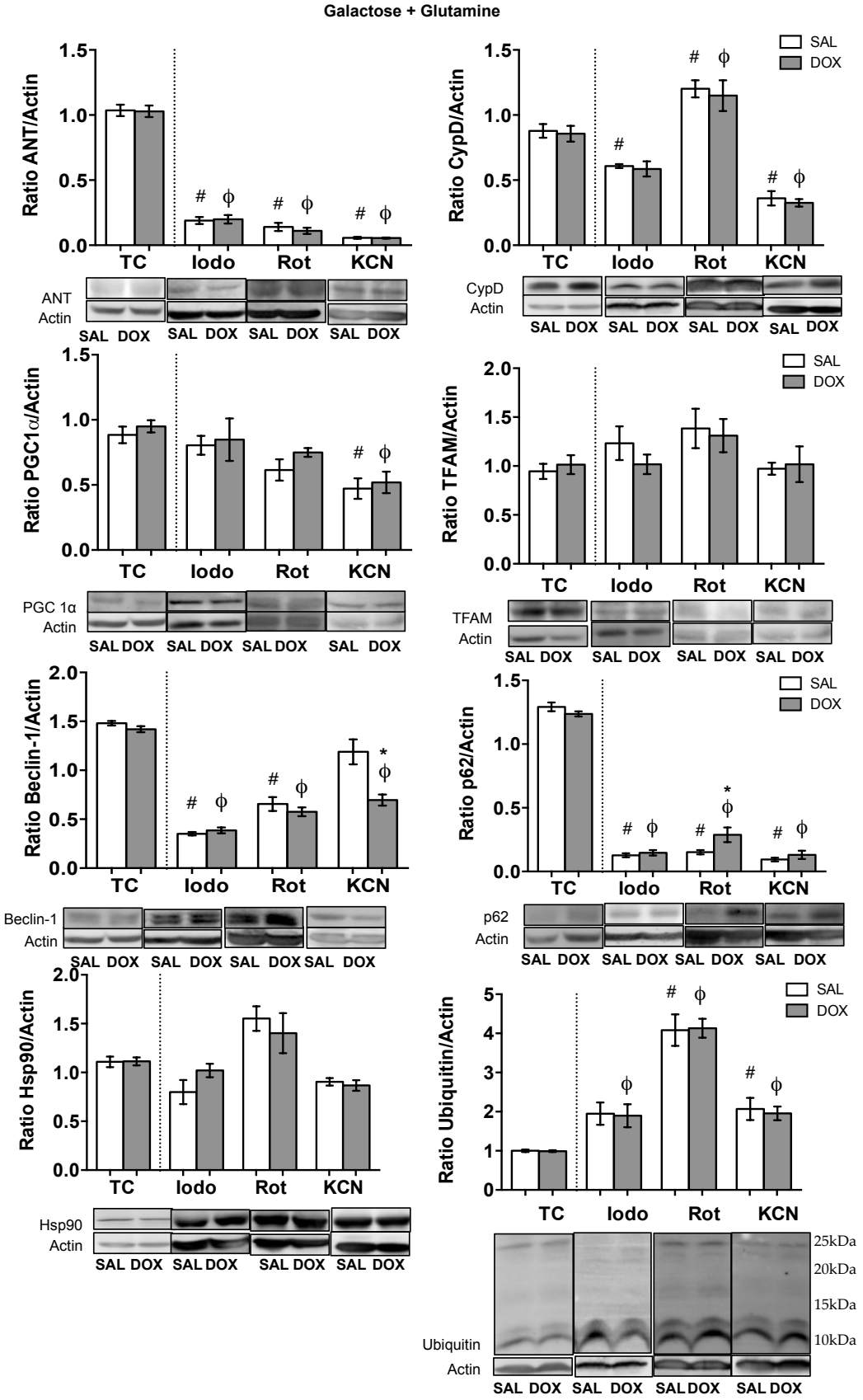

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Figure 40. *Proteins involved in the mitochondria events probed in galactose plus glutamine perfused hearts from SAL or DOX-treated rats.*
*Protein was harvested after time control perfusion (TC) and after inhibitor titration. Representative Western blot for the mentioned proteins together with their respective loading control. For ubiquitin quantification, a larger area was quantified for each group. The representative blot allows for a comparison of SAL and DOX groups for each condition. They should not be used as representative comparison between TC and inhibitor-treated samples. These were performed in different membranes, although all normalized to actin of the respective sample. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. . Comparisons of TC group vs. heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=4-6), # p < 0.05 TC SAL vs. inhibitor SAL, Φ p < 0.05 TC DOX vs. inhibitor DOX, * p < 0.05 SAL vs. DOX.*
Abbreviations: *ANT (33kDa)-Adenine nucleotide translocator; CypD (18kDa)-Cyclophilin D; PGC-1α (113kDa)-Peroxisome proliferator-activated receptor-gamma coactivator; TFAM (25kDa)-Mitochondrial transcription factor A; Beclin- 1 (60kDa); p62 (62kDa); Hsp90 (90kDa)-Heat shock protein 90; Ubiquitin (10kDa); Actin (43kDa). TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide*

4.2.4 mRNA alterations during sub-chronic DOX treatment

Regarding the previously Western blotting results, our next objective was to find out whether transcripts were altered in the presence of DOX.

4.2.4.1 mRNA content in NP sub-chronic hearts

Figure 41 shows the comparisons between SAL vs. DOX NP hearts. We measured a significant decrease for ANT and HIF- 1α transcripts in DOX vs. SAL hearts.

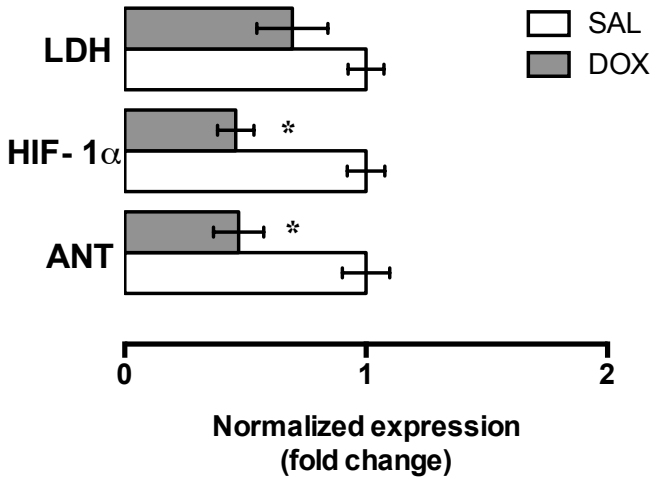

Figure 41. *mRNA content on sub-chronically treated non-perfused hearts.*
*mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test, data are mean ± SEM, (n=3-4), * p < 0.05 SAL vs. DOX.*
Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase.*

4.2.4.2 Transcripts in SAL or DOX sub-chronic treated hearts perfused with glucose: alterations with inhibitors

Hearts perfused with glucose (TC) were compared with hearts after titration perfusion (Fig. 42). For ANT mRNA content, a decrease in the DOX heart vs. SAL counterpart was observed in the presence of KCN. Regarding HIF- 1α mRNA, a decrease in DOX vs. SAL hearts was measured in TC hearts and after KCN titration. Interestingly, a very large increase in HIF- 1α transcript was observed in DOX hearts when titrated with iodoacetate. The difference was significant when comparing with the SAL counterpart and with the DOX TC heart. When LDH mRNA was evaluated, a DOX vs. SAL decrease was observed in TC, iodoacetate and rotenone perfusion conditions, while the same transcript was increased in DOX hearts titrated with KCN vs. the respective TC (Fig. 42).

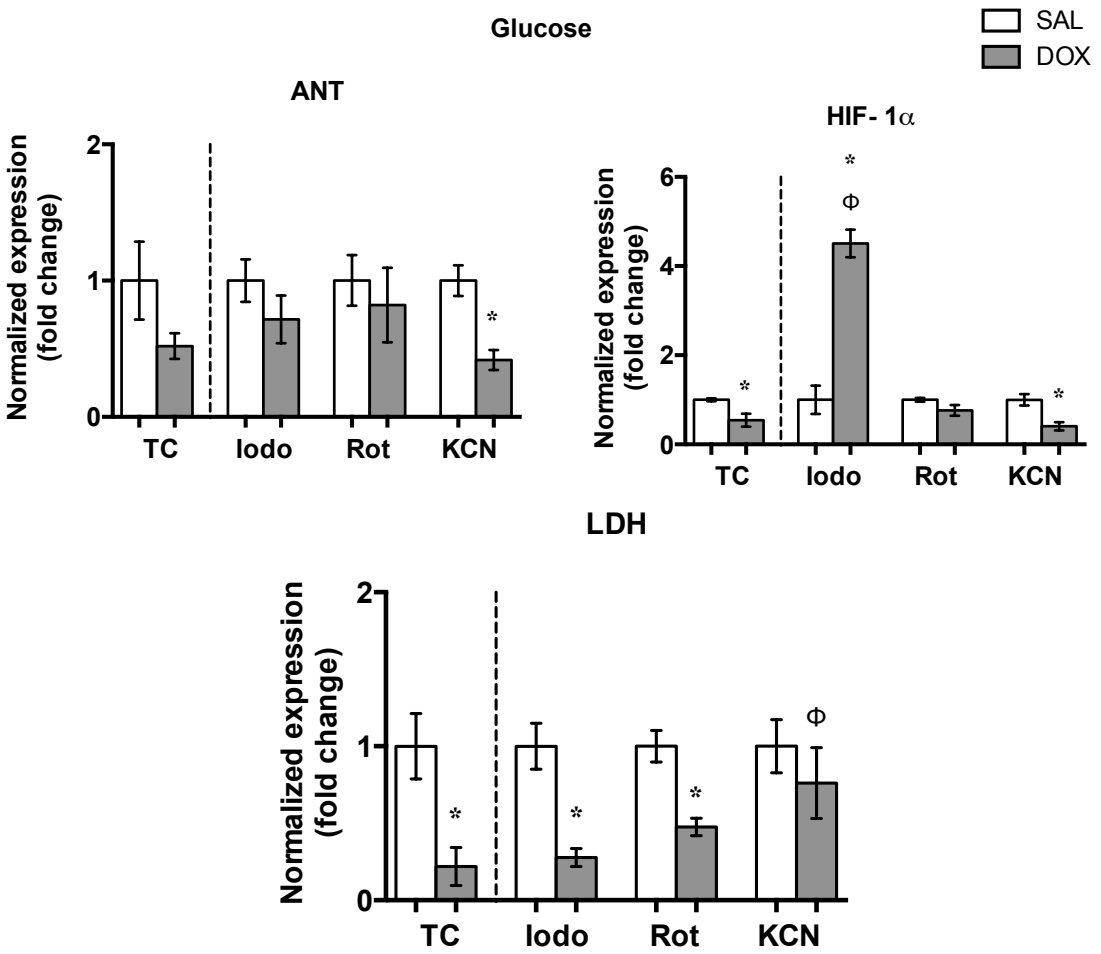

Figure 42. *mRNA content on sub-chronically treated hearts and perfused with glucose (TC) and glucose with inhibitors.*

*mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of DOX heart TC group vs. DOX heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=3-4), Φ p < 0.05 TC DOX vs. inhibitor DOX, * p < 0.05 SAL vs. DOX.*

Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α-Hypoxia-inducible factor 1-alpha ; LDH-Lactate dehydrogenase. TC-Time Control; Iodo-Iodoacetate; Rot-Rotenone; KCN-Potassium cyanide.*

4.2.4.3 Transcripts in SAL or DOX sub-chronic treated hearts perfused with galactose plus glutamine: alterations with inhibitors

Using galactose plus glutamine as perfusate (Fig. 43) we identified alterations on mRNA content for TC hearts for ANT with an increase on DOX hearts, while no alterations were observed in hearts perfused in the presence of inhibitors. For HIF-1α transcript we observed a significant decrease in DOX hearts perfused in the presence of iodoacetate and rotenone. Also, for DOX hearts perfused with rotenone a decline of LDH content was detected, while in the presence of other inhibitor no differences were established between treatments. However, comparing TC DOX hearts with DOX hearts in the presence of inhibitors, a significant decrease of ANT transcript was observed for all the three inhibitors used (Fig. 43).

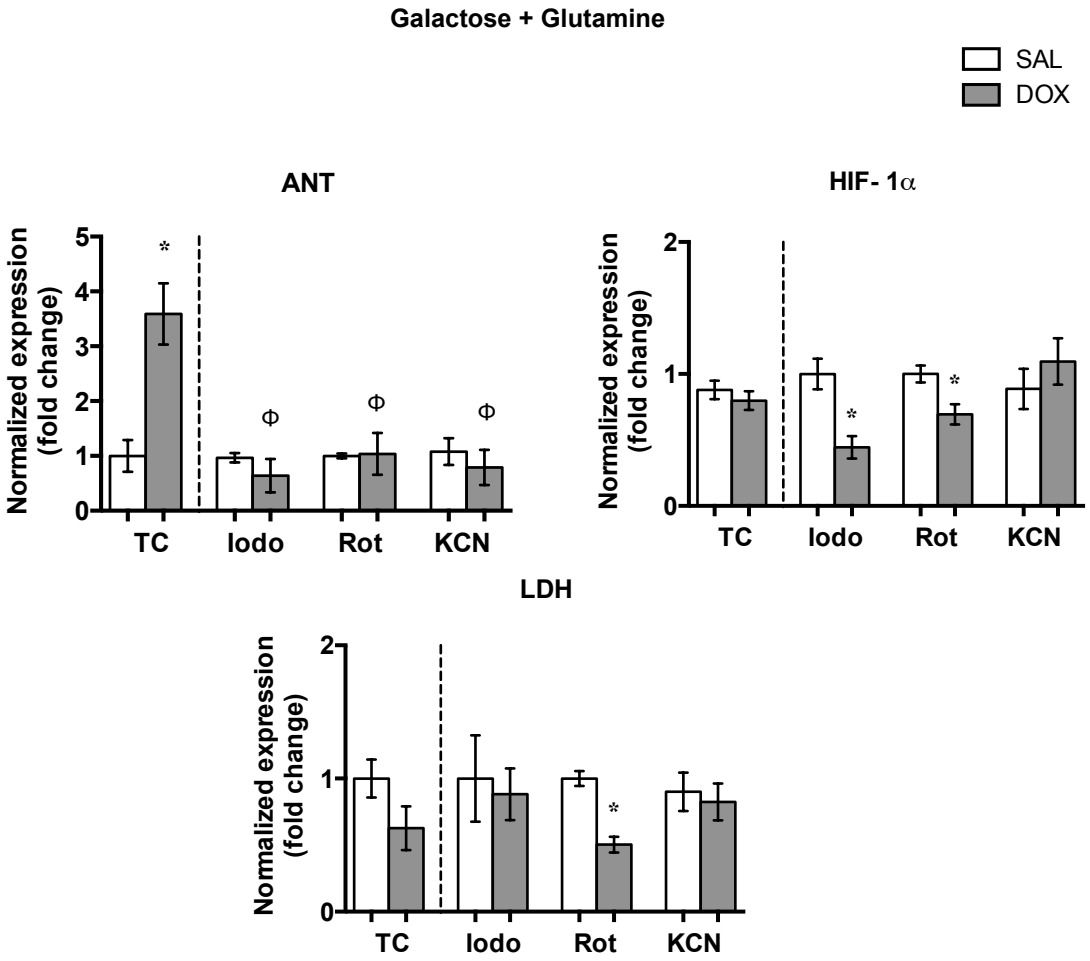

Figure 43. *mRNA content on hearts sub-chronically treated and perfused with galactose plus glutamine (TC) and galactose plus glutamine with inhibitors.*

*mRNA levels were normalized to Actin, results were calculated by ΔΔCT method. Comparisons between SAL and DOX group for the same condition were performed using a Student´s t-test. Comparisons of DOX heart TC group vs. DOX heart perfusion condition were performed with one-way ANOVA, followed by the Bonferroni post-test, data are mean ± SEM, (n=3-4), Φ p < 0.05 TC DOX vs. inhibitor DOX, * p < 0.05 SAL vs. DOX.*

Abbreviations: *ANT-Adenine nucleotide translocator; Hif-1α- Hypoxia-inducible factor 1-alpha ; LDH- Lactate dehydrogenase. TC- Time Control; Iodo- iodoacetate; Rot- rotenone; KCN- potassium cyanide.*

4.2.5 Discussion of sub-chronic treatment results

Heart failure originates from an unbalance between the demand of the organism and the capacity of the heart to perform his work. This unbalance may result from decreased oxygen and substrate handling caused by mitochondrial defects induced by genetic or toxic factors, among others [293]. A less than competent ATP formation leads to an inefficient energy use, resulting from increased workload to the myocardium following hypertension or even altered cardiac structure [245].

During chronic cardiac stress, such as DOX administration, the heart initiates an adaptive process that comprises left ventricular hypertrophy, and functional and metabolic remodeling as describe before for the acute treatment protocol. However, metabolic adaptation can become insufficient with a lower capacity to oxidize glucose leading to decreased efficiency and loss of cardiac function [294]. Impaired substrate metabolism can contribute to contractile dysfunction and cardiac remodeling which is a characteristic of heart failure.

To explore the different aspects of DOX-induced metabolic remodeling, we used one acute and one sub-chronic treatment protocol. The sub-chronic protocol would mimic the upcoming consequences of a bout of treatment course on heart metabolism. We used three distinct metabolic fuels and three inhibitors to try to pinpoint alterations in cardiac metabolism that otherwise would remain silent under basal conditions.

In an attempt to increase the knowledge regarding DOX-induced selective cardiotoxicity, we next performed a protocol for DOX sub-chronic treatment (7+1

weeks) [88] on Wistar rats. In terms of animal data, the results demonstrated that animals decrease their rate of body weight without any alterations on heart mass, confirming previous reports using the same protocol [88,269].

Taking into account that cardiac metabolism regulates contractibility [245], the author's objective was to induce metabolic changes in heart in order to detect if DOX could alter cardiac metabolic behavior. Previous evidence suggest that contractile performance is improved when the heart relies more in the oxidation of glucose and lactate in alternative to fatty acids [271]. By observing the hemodynamic results obtaining in the sub-chronic treatment protocol, SAL and DOX hearts perfused with different substrates in the presence of the different inhibitors showed differential responses during perfusion. In this protocol, perfusion with octanoate plus malate was not performed due to financial and time constrains. During perfusion with glucose and with iodoacetate as inhibitor, DOX hearts showed an improved heart rate when compared with SAL hearts. This suggests that most likely the glycolytic pathway is up-regulated in the hearts of DOX-treated rats, explaining the lower effect of the GAPDH inhibitor [186,244]. However, both hearts showed equal response to the inhibitor in terms of the RPP. The presence of rotenone or KCN in perfusate did not result in any difference between treatments, indicating that DOX hearts have the same susceptibility as SAL hearts to mitochondrial inhibitors under the condition of glucose perfusion. By perfusing hearts with galactose plus glutamine, the TCA cycle was directly fed substrates, decreasing the weight of glycolytic pathway in hearts treated with SAL or DOX Since galactose does not undergo glycolytic-based metabolism in the absence of glucose [239], we expect that the vast majority of cardiac metabolism is based on OXPHOS-induced ATP

generation. The presence of iodoacetate induced a decline of heart rate in DOX hearts, with significant differences in the recovery phase, when comparing with SAL hearts, however this effect is not observed in RPP, when both SAL and DOX hearts presented similar responses. Since these results are the opposite of what occurred in the acute protocol treatment, where DOX demonstrated to handle better iodoacetate inhibition. Facing this difference, we suggest that after a treatment protocol, the compensatory mechanisms observed in the acute study are not sufficient to maintain energy production and cardiac function, especially during metabolic stress. The addition of rotenone or KCN also decreased both hemodynamic rates but in this case no significant differences between SAL hearts and DOX hearts were detected, which may suggest that at least in this treatment protocol, no major differences between SAL and DOX regarding OXPHOS inhibition exist.

Concerning the different metabolic adaptations in heart, it is important that an existing protein modification exerts a key regulatory role in metabolism. When metabolic enzymes are redundant or expressed in reserve would not be able to regulate flux through the pathway [245]. It is therefore possible to have dramatic changes in expression of some proteins without much effect on substrate flux. In other words, changes in flux cannot simply be inferred from changes in enzyme activity or expression.

However, our results show that after perfusion with different substrates and with addition of different inhibitors, differences in the content of several proteins were detected. Trying to justify the results obtained during perfusion, selected proteins related with glycolysis or mitochondria were measured on SAL and DOX hearts. On

non-perfused hearts, hexokinase I was increased in DOX hearts. Since hexokinase I is a regulator of cell death [295,296] as well as a limiting step in glycolysis [297] it is likely that increased glycolytic fluxes in DOX hearts may result, at least partly, from an increase in the content of this protein.

In hearts perfused with glucose and different inhibitors, glycolytic protein alterations were not observed for the three inhibitors when SAL-treated hearts were compared with DOX-treated hearts suggesting similar mechanism if down-regulation following metabolic failure. Other proteins investigated showed similar results, with the exception of p62 protein. In this case, DOX hearts perfused in the presence of iodoacetate, showed increase protein content, suggesting inhibition of clearance events. In hearts perfused with galactose plus glutamine and inhibitors, glycolytic proteins did not have any alterations on DOX hearts comparing with SAL hearts; however, DOX hearts showed a decrease on Beclin-1 when KCN was present in the perfusate and an significant increase on p62 when hearts were perfused with rotenone, which again suggest a possible caused by DOX.

Differences were detected in both SAL and DOX hearts when TC hearts of respective perfusion substrate were compared with hearts perfused in the presence of inhibitors for each substrate. With glucose in the perfusion buffer, significant differences were found in hexokinase II and PDH, whereas in the presence of the three inhibitors, both SAL and DOX hearts showed an increase of hexokinase II and a decrease of PDH comparing with TC hearts. A possible explanation is that in the presence of inhibitors, the heart may try to compensate the bioenergetics failure by increasing hexokinase II expression. The meaning of the decrease in PDH is not clear. For other

glycolytic proteins, GAPDH was increased in DOX hearts in the presence of iodoacetate, which, similarly to the acute treatment study, may be the direct result of iodoacetate-induced enzyme inhibition. The idea that metabolic inhibition can cause fast (~ 40 minutes) in the content of metabolic enzymes is seen in other proteins after perfusion with the different inhibitors. Regarding proteins relevant for mitochondrial quality control, a decrease of PGC 1α, CypD, p62 and Hsp90 proteins was detected in both SAL and DOX hearts in the presence of all inhibitors, confirming that the bioenergetics collapse decreases proteins responsible for the maintenance of mitochondrial mass. However, it appears that apoptotic and autophagy proteins can still be present to trigger cell death [287,288]. Our own data for ubiquitin appears to suggest a decrease when inhibitors are present, regardless of the treatment group, being a possible indication that metabolic inhibition leads to increased clearance of ubiquinated proteins. In hearts perfused with galactose plus glutamine, hexokinase II, PKM 1/2 and LDHA showed a decrease in protein amount in both SAL and DOX hearts when compared with TC hearts, these results suggest that the presence of inhibitors again lead to an overall down-regulation of protein compositions.

When hearts were perfused with galactose plus glutamine, ANT, Beclin- 1 and p62 decreased in both SAL and DOX hearts when the inhibitors were present, while ubiquitin increased in both SAL and DOX hearts, which implies an accumulation of tagged proteins and possibly altered autophagy [298]. One interesting possibility is that this may lead to cardiac aging and hence explain the development of cardiomyopathies later in life. Interestingly, CypD protein increased in both SAL and DOX hearts when rotenone was used, but decreased when KCN was used instead in the perfusion buffer. The difference between KCN and rotenone in this and in other

perfusion protocols may have been related by their mode of action. Rotenone is known to induce ROS production by inhibiting complex I [247], which is not as evident when KCN is used. Oxidation of proteins by complex I-produced ROS may result in the activation of distinct stress responses, which may also involve the triggering of the mitochondrial permeability transition pore, as reported by previous results from our group [54,104]. Also, PGC 1α is decrease in both hearts when KCN was added, suggesting an impairment of mitochondrial biogenesis responses during respiratory chain inhibition [299].

An evaluation of mRNA that code proteins that influence glycolysis, such as HIF- 1α and LDH [289], as well as the ANT, was performed to understand if protein alterations were related with mRNA differences or instead result from differential degradation. On non-perfused hearts, HIF- 1α and ANT mRNA was decreased on DOX hearts, again consistent with previous reports [290,300]. Meanwhile, when glucose perfused hearts were analyzed, a decrease of ANT mRNA content was observed in DOX hearts perfused with KCN, for HIF- 1 α transcript DOX TC hearts and DOX hearts shows a decrease in the presence of KCN, this result is consistent with previous studies [290,300]. However, for DOX hearts perfused in iodoacetate HIF- 1α mRNA levels were increased, which is in agreement with a possible DOX-induced stimulation of glycolysis, based on HIF- 1α-up-regulation [301]. This is supported by increased glycolytic proteins in in DOX hearts perfused with iodoacetate, such as hexokinase II, GAPDH and LDHA. For LDH mRNA content, results were consistent with the TC group, with iodoacetate and rotenone showing a decrease comparing with SAL hearts. Interestingly, it is known that down regulation of LDH activity have an impact on mitochondria bioenergetics [302], however these

result on mRNA levels were not followed by concomitant alterations in protein content. For galactose plus glutamine perfusion on DOX TC hearts, ANT mRNA was increased, contrarily to results in the presence of inhibitors, which did not reveal differences between treatments. An immediate explanation for this is not available, hence more studies need to be done to take further conclusion. The transcript for HIF- 1α was decreased in DOX hearts when iodoacetate and rotenone were added in perfusate buffer, again these results are not consistent with differences in glycolytic proteins amount among treatments, demonstrated that inhibitors did not induce any modification on DOX hearts, since NP hearts showed the same effect [290,300].

Although not allowing conclusive explanations regarding the detection of clear sub-clinical mitochondrial metabolic defects, these results confirm that our experimental protocol is effective in pinpointing metabolic defects under drug-induced disease or even the pathophysiology of cardiovascular diseases.

In summary the data from the sub-chronic treatment with DOX, the results suggest that an increased glycolytic phenotype is observed, which explains the higher resistance to iodoacetate. Consistent with the results obtained in the acute treatment also proteins related with mitochondrial biogenesis were decreased, while results from autophagic proteins suggest that an inhibition of the autophagic process may be occurring in DOX-treated rats. Both alterations may have long-term effects in the heart.

Chapter 5: General Conclusion

Doxorubicin is an antibiotic belonging to the anthracycline family and is well known for its anti-neoplastic activity, being for decades in the treatment of several types of cancer. Severe side effects were described during treatment with DOX, including a significant incidence of cardiovascular complications, leading to heart failure [36,51,52]. However, the main mechanism of DOX toxicity associated to a dose-dependent and cumulative cardiomyopathy are not yet entirely clear.

In the present thesis, the objective was to use of model of metabolic inhibition in perfused hearts from saline (SAL) and DOX-treated rats in order to identify metabolic alterations caused by an acute and sub-chronic treatment, including mitochondrial deficits that under basal conditions have no impact on hemodynamic function. To achieve the objective we performed an acute and a sub-chronic protocol, and our strategy involved using metabolic inhibitors titrate SAL and DOX heart function under different perfusion conditions. Three distinct inhibitors, one glycolytic (iodoacetate) and two mitochondrial (rotenone and KCN) were used.

To be best of our knowledge, this was the first time this perfusion protocol was performed to pinpoint metabolic defects in the heart. Although we could not confirm a clear mitochondrial defect in DOX-treated hearts that would compromise cardiac hemodynamic performance in the presence of inhibitors, we observed what appears a more robust glycolytic component in hearts from DOX-treated rats. This can be seen as an adaptation to the toxicity of DOX. Since we have previously demonstrated cardiac mitochondrial defects in DOX-treated rats [54,88], it is likely that other metabolic adaptations in treated hearts may hide the already described deficits, which were not disclosed by our protocol of inhibitor titration.

Also, another novelty is that the present work demonstrated for the first time variations in proteins involved in metabolism, mitochondrial and a autophagy which not only occurred after DOX treatment but also differed in each group after 40 minutes of perfusion with different inhibitors. Interestingly, one of those defects involved a decrease in the ANT, which was already described in another of our publications [86]. This defect may alter the mitochondrial capacity to export ATP, contributing to a decreased hemodynamic performance. Finally, of note is what appears to be a defective autophagic and ubiquitination fluxes after DOX treatment, suggesting that accumulation of defective intracellular structures may result from the treatment, mimicking a condition of cardiac aging.

In the opinion of the author more studies need to be performed in order to confirm how DOX modulate the different metabolic pathways and how that changes as time post-treatment progresses. This should be performed with longer treatments or at least with animals isolated after a longer elapsed time post-treatment.

Future lines of study:

1. Investigate rates of glycolytic flux in hearts during perfusion in order to determinate the metabolic action of inhibitors.
2. Further analyses of protein and enzymatic activity related with heart metabolic pathways after SAL and DOX treatments, as well as measurement of adenine nucleotides.

Chapter 6: Bibliography

1. Liesa M, Palacin M, Zorzano A (2009) Mitochondrial dynamics in mammalian health and disease. Physiol Rev 89: 799-845.

2. Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, Rossignol R (2007) Mitochondrial bioenergetics and structural network organization. J Cell Sci 120: 838-848.

3. Pereira CV, Moreira AC, Pereira SP, Machado NG, Carvalho FS, Sardao VA, Oliveira PJ (2009) Investigating drug-induced mitochondrial toxicity: a biosensor to increase drug safety? Curr Drug Saf 4: 34-54.

4. Kroemer G (1999) Mitochondrial control of apoptosis: an overview. Biochem Soc Symp 66: 1-15.

5. Johannsen TH, Kamstrup PR, Andersen RV, Jensen GB, Sillesen H, Tybjaerg-Hansen A, Nordestgaard BG (2009) Hepatic lipase, genetically elevated high-density lipoprotein, and risk of ischemic cardiovascular disease. J Clin Endocrinol Metab 94: 1264-1273.

6. Rasola A, Bernardi P (2011) Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium 50: 222-233.

7. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E (1998) Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. FEBS Lett 424: 155-158.

8. Gaude E, Frezza C (2014) Defects in mitochondrial metabolism and cancer. Cancer Metab 2: 10.

9. Erecinska M, Wilson DF (1982) Regulation of cellular energy metabolism. J Membr Biol 70: 1-14.

10. Romano AH, Conway T (1996) Evolution of carbohydrate metabolic pathways. Res Microbiol 147: 448-455.

11. Miao P, Sheng S, Sun X, Liu J, Huang G (2013) Lactate dehydrogenase A in cancer: a promising target for diagnosis and therapy. IUBMB Life 65: 904-910.

12. Ventura-Clapier R, Garnier A, Veksler V (2004) Energy metabolism in heart failure. J Physiol 555: 1-13.

13. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R (2003) Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. J Physiol 551: 491-501.

14. Scarpulla RC (2002) Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. Gene 286: 81-89.

15. Shi Y, Dierckx A, Wanrooij PH, Wanrooij S, Larsson NG, Wilhelmsson LM, Falkenberg M, Gustafsson CM (2012) Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. Proc Natl Acad Sci U S A 109: 16510-16515.

16. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms

controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98: 115-124.

17. Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP (2000) Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest 106: 847-856.

18. Bonadonna G, Monfardini S, De Lena M, Fossati-Bellani F (1969) Clinical evaluation of adriamycin, a new antitumour antibiotic. Br Med J 3: 503-506.

19. Bonadonna G, Monfardini S, De Lena M, Fossati-Bellani F, Beretta G (1970) Phase I and preliminary phase II evaluation of adriamycin (NSC 123127). Cancer Res 30: 2572-2582.

20. Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS, Moreira PI (2009) Doxorubicin: the good, the bad and the ugly effect. Curr Med Chem 16: 3267-3285.

21. Hortobagyi GN (1997) Anthracyclines in the treatment of cancer. An overview. Drugs 54 Suppl 4: 1-7.

22. Aubel-Sadron G, Londos-Gagliardi D (1984) Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. Biochimie 66: 333-352.

23. Singal PK, Iliskovic N (1998) Doxorubicin-induced cardiomyopathy. N Engl J Med 339: 900-905.

24. Villani F, Beretta G, Guindani A (1979) Evaluation of early doxorubicin-induced cardiotoxicity by means of systolic time intervals. Cancer Chemother Pharmacol 3: 249-251.

25. Bristow MR, Billingham ME, Mason JW, Daniels JR (1978) Clinical spectrum of anthracycline antibiotic cardiotoxicity. Cancer Treat Rep 62: 873-879.

26. Chatterjee K, Zhang J, Honbo N, Karliner JS (2010) Doxorubicin cardiomyopathy. Cardiology 115: 155-162.

27. Horenstein MS, Vander Heide RS, L'Ecuyer TJ (2000) Molecular basis of anthracycline-induced cardiotoxicity and its prevention. Mol Genet Metab 71: 436-444.

28. Frishman WH, Sung HM, Yee HC, Liu LL, Keefe D, Einzig AI, Dutcher J (1997) Cardiovascular toxicity with cancer chemotherapy. Curr Prob Cancer 21: 301-360.

29. Lefrak EA, Pitha J, Rosenheim S, Gottlieb JA (1973) A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer 32: 302-314.

30. Pai VB, Nahata MC (2000) Cardiotoxicity of chemotherapeutic agents: incidence, treatment and prevention. Drug Saf 22: 263-302.

31. Pereira GC, Silva AM, Diogo CV, Carvalho FS, Monteiro P, Oliveiraalo PJ (2011) Drug-induced cardiac mitochondrial toxicity and protection: from doxorubicin to carvedilol. Curr Pharm Des 17: 2113-2129.

32. Alexander J, Dainiak N, Berger HJ, Goldman L, Johnstone D, Reduto L, Duffy T, Schwartz P, Gottschalk A, Zaret BL (1979) Serial assessment of doxorubicin cardiotoxicity with quantitative radionuclide angiocardiography. The New England journal of medicine 300: 278-283.

33. Buzdar AU, Marcus C, Smith TL, Blumenschein GR (1985) Early and delayed clinical cardiotoxicity of doxorubicin. Cancer 55: 2761-2765.

34. Yeh ET, Tong AT, Lenihan DJ, Yusuf SW, Swafford J, Champion C, Durand JB, Gibbs H, Zafarmand AA, Ewer MS (2004) Cardiovascular complications of cancer therapy: diagnosis, pathogenesis, and management. Circulation 109: 3122-3131.

35. Wallace KB (2003) Doxorubicin-induced cardiac mitochondrionopathy. Pharmacol Toxicol 93: 105-115.

36. Christiansen S, Autschbach R (2006) Doxorubicin in experimental and clinical heart failure. Eur J Cardiothorac Surg 30: 611-616.

37. Jain D (2000) Cardiotoxicity of doxorubicin and other anthracycline derivatives. J Nucl Cardiol 7: 53-62.

38. Steinherz LJ, Steinherz PG, Tan CT, Heller G, Murphy ML (1991) Cardiac toxicity 4 to 20 years after completing anthracycline therapy. JAMA 266: 1672-1677.

39. Lipshultz SE, Lipsitz SR, Mone SM, Goorin AM, Sallan SE, Sanders SP, Orav EJ, Gelber RD, Colan SD (1995) Female sex and drug dose as risk

factors for late cardiotoxic effects of doxorubicin therapy for childhood cancer. The New England journal of medicine 332: 1738-1743.

40. Danesi R, Fogli S, Gennari A, Conte P, Del Tacca M (2002) Pharmacokinetic-pharmacodynamic relationships of the anthracycline anticancer drugs. Clin Pharmacokinet 41: 431-444.

41. Lipshultz SE (2006) Exposure to anthracyclines during childhood causes cardiac injury. Seminars in Oncology 33: S8-14.

42. Kremer LC, van Dalen EC, Offringa M, Voute PA (2002) Frequency and risk factors of anthracycline-induced clinical heart failure in children: a systematic review. Ann Oncol 13: 503-512.

43. Doyle JJ, Neugut AI, Jacobson JS, Grann VR, Hershman DL (2005) Chemotherapy and cardiotoxicity in older breast cancer patients: a population-based study. J Clin Oncol 23: 8597-8605.

44. De Angelis A, Piegari E, Cappetta D, Marino L, Filippelli A, Berrino L, Ferreira-Martins J, Zheng H, Hosoda T, Rota M, Urbanek K, Kajstura J, Leri A, Rossi F, Anversa P (2010) Anthracycline cardiomyopathy is mediated by depletion of the cardiac stem cell pool and is rescued by restoration of progenitor cell function. Circulation 121: 276-292.

45. Lipshultz SE, Rifai N, Dalton VM, Levy DE, Silverman LB, Lipsitz SR, Colan SD, Asselin BL, Barr RD, Clavell LA, Hurwitz CA, Moghrabi A, Samson Y, Schorin MA, Gelber RD, Sallan SE (2004) The effect of

dexrazoxane on myocardial injury in doxorubicin-treated children with acute lymphoblastic leukemia. N Engl J Med 351: 145-153.

46. Zhu W, Shou W, Payne RM, Caldwell R, Field LJ (2008) A mouse model for juvenile doxorubicin-induced cardiac dysfunction. Pediatr Res 64: 488-494.

47. Cusack BJ, Musser B, Gambliel H, Hadjokas NE, Olson RD (2003) Effect of dexrazoxane on doxorubicin pharmacokinetics in young and old rats. Cancer Chemother Pharmacol 51: 139-146.

48. Robert J, Hoerni B (1983) Age dependence of the early-phase pharmacokinetics of doxorubicin. Cancer research 43: 4467-4469.

49. Li J, Gwilt PR (2003) The effect of age on the early disposition of doxorubicin. Cancer Chemother Pharmacol 51: 395-402.

50. Hong YM, Kim HS, Yoon HR (2002) Serum lipid and fatty acid profiles in adriamycin-treated rats after administration of L-carnitine. Pediatr Res 51: 249-255.

51. Singal PK, Li T, Kumar D, Danelisen I, Iliskovic N (2000) Adriamycin-induced heart failure: mechanism and modulation. Mol Cell Biochem 207: 77-86.

52. Takemura G, Fujiwara H (2007) Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Prog Cardiovasc Dis 49: 330-352.

53. Billingham ME, Mason JW, Bristow MR, Daniels JR (1978) Anthracycline cardiomyopathy monitored by morphologic changes. Cancer Treat Rep 62: 865-872.

54. Oliveira PJ, Bjork JA, Santos MS, Leino RL, Froberg MK, Moreno AJ, Wallace KB (2004) Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. Toxicol Appl Pharmacol 200: 159-168.

55. Tokarska-Schlattner M, Zaugg M, Zuppinger C, Wallimann T, Schlattner U (2006) New insights into doxorubicin-induced cardiotoxicity: the critical role of cellular energetics. J Mol Cell Cardiol 41: 389-405.

56. Unverferth DV, Magorien RD, Unverferth BP, Talley RL, Balcerzak SP, Baba N (1981) Human myocardial morphologic and functional changes in the first 24 hours after doxorubicin administration. Cancer Treat Rep 65: 1093-1097.

57. Ascensao A, Magalhaes J, Soares JM, Ferreira R, Neuparth MJ, Marques F, Oliveira PJ, Duarte JA (2005) Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. Am J Physiol Heart Circ Physiol 289: H722-731.

58. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56: 185-229.

59. Sardao VA, Oliveira PJ, Holy J, Oliveira CR, Wallace KB (2009) Morphological alterations induced by doxorubicin on H9c2 myoblasts: nuclear, mitochondrial, and cytoskeletal targets. Cell Biol Toxicol 25: 227-243.

60. Ferreira AL, Matsubara LS, Matsubara BB (2008) Anthracycline-induced cardiotoxicity. Cardiovasc Hematol Agents Med Chem 6: 278-281.

61. Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57: 727-741.

62. Mukhopadhyay P, Rajesh M, Batkai S, Kashiwaya Y, Hasko G, Liaudet L, Szabo C, Pacher P (2009) Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. Am J Physiol Heart Circ Physiol 296: H1466-1483.

63. DeAtley SM, Aksenov MY, Aksenova MV, Jordan B, Carney JM, Butterfield DA (1999) Adriamycin-induced changes of creatine kinase activity in vivo and in cardiomyocyte culture. Toxicology 134: 51-62.

64. Lebrecht D, Kokkori A, Ketelsen UP, Setzer B, Walker UA (2005) Tissue-specific mtDNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. J Pathol 207: 436-444.

65. Cardoso S, Santos RX, Carvalho C, Correia S, Pereira GC, Pereira SS, Oliveira PJ, Santos MS, Proenca T, Moreira PI (2008) Doxorubicin

increases the susceptibility of brain mitochondria to Ca(2+)-induced permeability transition and oxidative damage. Free Radic Biol Med 45: 1395-1402.

66. Machado NG, Baldeiras I, Pereira GC, Pereira SP, Oliveira PJ (2010) Sub-chronic administration of doxorubicin to Wistar rats results in oxidative stress and unaltered apoptotic signaling in the lung. Chem Biol Interact 188: 478-486.

67. Ascensao A, Lumini-Oliveira J, Machado NG, Ferreira RM, Goncalves IO, Moreira AC, Marques F, Sardao VA, Oliveira PJ, Magalhaes J (2011) Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. Clin Sci (Lond) 120: 37-49.

68. Santos DL, Moreno AJ, Leino RL, Froberg MK, Wallace KB (2002) Carvedilol protects against doxorubicin-induced mitochondrial cardiomyopathy. Toxicol Appl Pharmacol 185: 218-227.

69. Outomuro D, Grana DR, Azzato F, Milei J (2007) Adriamycin-induced myocardial toxicity: new solutions for an old problem? Int J Cardiol 117: 6-15.

70. Kaiserova H, Simunek T, Sterba M, den Hartog GJ, Schroterova L, Popelova O, Gersl V, Kvasnickova E, Bast A (2007) New iron chelators in anthracycline-induced cardiotoxicity. Cardiovasc Toxicol 7: 145-150.

71. Quiles JL, Huertas JR, Battino M, Mataix J, Ramirez-Tortosa MC (2002) Antioxidant nutrients and adriamycin toxicity. Toxicology 180: 79-95.

72. Doroshow JH, Locker GY, Myers CE (1980) Enzymatic defenses of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin. J Clin Invest 65: 128-135.

73. Li T, Danelisen I, Singal PK (2002) Early changes in myocardial antioxidant enzymes in rats treated with adriamycin. Molecular and cellular biochemistry 232: 19-26.

74. Thornalley PJ, Dodd NJ (1985) Free radical production from normal and adriamycin-treated rat cardiac sarcosomes. Biochemical pharmacology 34: 669-674.

75. Doroshow JH (1983) Anthracycline antibiotic-stimulated superoxide, hydrogen peroxide, and hydroxyl radical production by NADH dehydrogenase. Cancer Res 43: 4543-4551.

76. Doroshow JH, Davies KJ (1986) Redox cycling of anthracyclines by cardiac mitochondria. II. Formation of superoxide anion, hydrogen peroxide, and hydroxyl radical. J Biol Chem 261: 3068-3074.

77. Doroshow JH (1983) Effect of anthracycline antibiotics on oxygen radical formation in rat heart. Cancer research 43: 460-472.

78. Vasquez-Vivar J, Martasek P, Hogg N, Masters BS, Pritchard KA, Jr., Kalyanaraman B (1997) Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. Biochemistry 36: 11293-11297.

79. Demant EJ, Jensen PK (1983) Destruction of phospholipids and respiratory-chain activity in pig-heart submitochondrial particles induced by an adriamycin-iron complex. Eur J Biochem 132: 551-556.

80. Marcillat O, Zhang Y, Davies KJ (1989) Oxidative and non-oxidative mechanisms in the inactivation of cardiac mitochondrial electron transport chain components by doxorubicin. Biochem J 259: 181-189.

81. Yoon SB, Kajiyama K, Hino Y, Sugiyama M, Ogura R (1983) Effect of adriamycin on lipid peroxide, glutathione peroxidase and respiratory responses of mitochondria from the heart, liver and kidney. Kurume Med J 30: 1-4.

82. Parker MA, King V, Howard KP (2001) Nuclear magnetic resonance study of doxorubicin binding to cardiolipin containing magnetically oriented phospholipid bilayers. Biochim Biophys Acta 1514: 206-216.

83. Goormaghtigh E, Huart P, Praet M, Brasseur R, Ruysschaert JM (1990) Structure of the adriamycin-cardiolipin complex. Role in mitochondrial toxicity. Biophys Chem 35: 247-257.

84. Claypool SM, Koehler CM (2012) The complexity of cardiolipin in health and disease. Trends Biochem Sci 37: 32-41.

85. Goormaghtigh E, Huart P, Brasseur R, Ruysschaert JM (1986) Mechanism of inhibition of mitochondrial enzymatic complex I-III by adriamycin derivatives. Biochim Biophys Acta 861: 83-94.

86. Oliveira PJ, Wallace KB (2006) Depletion of adenine nucleotide translocator protein in heart mitochondria from doxorubicin-treated rats--relevance for mitochondrial dysfunction. Toxicology 220: 160-168.

87. Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB (2001) Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. Cancer research 61: 771-777.

88. Pereira GC, Pereira SP, Pereira CV, Lumini JA, Magalhaes J, Ascensao A, Santos MS, Moreno AJ, Oliveira PJ (2012) Mitochondrionopathy phenotype in doxorubicin-treated Wistar rats depends on treatment protocol and is cardiac-specific. PLoS One 7: e38867.

89. Berthiaume JM, Wallace KB (2007) Adriamycin-induced oxidative mitochondrial cardiotoxicity. Cell Biol Toxicol 23: 15-25.

90. Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C (2002) Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. Cancer Res 62: 4592-4598.

91. Kuznetsov AV, Margreiter R, Amberger A, Saks V, Grimm M (2011) Changes in mitochondrial redox state, membrane potential and calcium precede mitochondrial dysfunction in doxorubicin-induced cell death. Biochim Biophys Acta 1813: 1144-1152.

92. Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB (2001) Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. Cancer Res 61: 771-777.

93. Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM (2012) p53 opens the mitochondrial permeability transition pore to trigger necrosis. Cell 149: 1536-1548.

94. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434: 652-658.

95. Baines CP (2009) The molecular composition of the mitochondrial permeability transition pore. J Mol Cell Cardiol 46: 850-857.

96. Javadov S, Karmazyn M, Escobales N (2009) Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. J Pharmacol Exp Ther 330: 670-678.

97. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 427: 461-465.

98. Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 9: 550-555.

99. Halestrap AP (2009) What is the mitochondrial permeability transition pore? J Mol Cell Cardiol 46: 821-831.

100. Leung AW, Varanyuwatana P, Halestrap AP (2008) The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. J Biol Chem 283: 26312-26323.

101. Baines CP (2009) The mitochondrial permeability transition pore and ischemia-reperfusion injury. Basic Res Cardiol 104: 181-188.

102. Halestrap AP, Pasdois P (2009) The role of the mitochondrial permeability transition pore in heart disease. Biochim Biophys Acta 1787: 1402-1415.

103. Liu J, Mao W, Ding B, Liang CS (2008) ERKs/p53 signal transduction pathway is involved in doxorubicin-induced apoptosis in H9c2 cells and cardiomyocytes. Am J Physiol Heart Circ Physiol 295: H1956-1965.

104. Oliveira PJ, Santos MS, Wallace KB (2006) Doxorubicin-induced thiol-dependent alteration of cardiac mitochondrial permeability transition and respiration. Biochemistry (Mosc) 71: 194-199.

105. Broekemeier KM, Dempsey ME, Pfeiffer DR (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 264: 7826-7830.

106. McStay GP, Clarke SJ, Halestrap AP (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the

mechanism of the mitochondrial permeability transition pore. Biochem J 367: 541-548.

107. Halestrap AP, Woodfield KY, Connern CP (1997) Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J Biol Chem 272: 3346-3354.

108. Tokarska-Schlattner M, Wallimann T, Schlattner U (2006) Alterations in myocardial energy metabolism induced by the anti-cancer drug doxorubicin. C R Biol 329: 657-668.

109. Berthiaume JM, Wallace KB (2007) Persistent alterations to the gene expression profile of the heart subsequent to chronic Doxorubicin treatment. Cardiovasc Toxicol 7: 178-191.

110. Richard C, Ghibu S, Delemasure-Chalumeau S, Guilland JC, Des Rosiers C, Zeller M, Cottin Y, Rochette L, Vergely C (2011) Oxidative stress and myocardial gene alterations associated with Doxorubicin-induced cardiotoxicity in rats persist for 2 months after treatment cessation. J Pharmacol Exp Ther 339: 807-814.

111. Ashley N, Poulton J (2009) Mitochondrial DNA is a direct target of anti-cancer anthracycline drugs. Biochem Biophys Res Commun 378: 450-455.

112. Adachi K, Fujiura Y, Mayumi F, Nozuhara A, Sugiu Y, Sakanashi T, Hidaka T, Toshima H (1993) A deletion of mitochondrial DNA in

murine doxorubicin-induced cardiotoxicity. Biochem Biophys Res Commun 195: 945-951.

113. Ellis CN, Ellis MB, Blakemore WS (1987) Effect of adriamycin on heart mitochondrial DNA. Biochem J 245: 309-312.

114. Hixon SC, Ellis CN, Daugherty JP (1981) Heart mitochondrial DNA synthesis: preferential inhibition by adriamycin. J Mol Cell Cardiol 13: 855-860.

115. Serrano J, Palmeira CM, Kuehl DW, Wallace KB (1999) Cardioselective and cumulative oxidation of mitochondrial DNA following subchronic doxorubicin administration. Biochim Biophys Acta 1411: 201-205.

116. Palmeira CM, Serrano J, Kuehl DW, Wallace KB (1997) Preferential oxidation of cardiac mitochondrial DNA following acute intoxication with doxorubicin. Biochim Biophys Acta 1321: 101-106.

117. Sardao VA, Oliveira PJ, Holy J, Oliveira CR, Wallace KB (2009) Doxorubicin-induced mitochondrial dysfunction is secondary to nuclear p53 activation in H9c2 cardiomyoblasts. Cancer Chemother Pharmacol 64: 811-827.

118. Singal PK, Iliskovic N, Li T, Kumar D (1997) Adriamycin cardiomyopathy: pathophysiology and prevention. FASEB J 11: 931-936.

119. Van Vleet JF, Ferrans VJ, Weirich WE (1980) Cardiac disease induced by chronic adriamycin administration in dogs and an evaluation of vitamin E and selenium as cardioprotectants. Am J Pathol 99: 13-42.

120. Yamanaka S, Tatsumi T, Shiraishi J, Mano A, Keira N, Matoba S, Asayama J, Fushiki S, Fliss H, Nakagawa M (2003) Amlodipine inhibits doxorubicin-induced apoptosis in neonatal rat cardiac myocytes. J Am Coll Cardiol 41: 870-878.

121. Spallarossa P, Garibaldi S, Altieri P, Fabbi P, Manca V, Nasti S, Rossettin P, Ghigliotti G, Ballestrero A, Patrone F, Barsotti A, Brunelli C (2004) Carvedilol prevents doxorubicin-induced free radical release and apoptosis in cardiomyocytes in vitro. J Mol Cell Cardiol 37: 837-846.

122. Myers CE, McGuire WP, Liss RH, Ifrim I, Grotzinger K, Young RC (1977) Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. Science 197: 165-167.

123. Mimnaugh EG, Siddik ZH, Drew R, Sikic BI, Gram TE (1979) The effects of alpha-tocopherol on the toxicity, disposition, and metabolism of adriamycin in mice. Toxicol Appl Pharmacol 49: 119-126.

124. Berthiaume JM, Oliveira PJ, Fariss MW, Wallace KB (2005) Dietary vitamin E decreases doxorubicin-induced oxidative stress without preventing mitochondrial dysfunction. Cardiovasc Toxicol 5: 257-267.

125. Zhang X, Peng X, Yu W, Hou S, Zhao Y, Zhang Z, Huang X, Wu K (2011) Alpha-tocopheryl succinate enhances doxorubicin-induced apoptosis

in human gastric cancer cells via promotion of doxorubicin influx and suppression of doxorubicin efflux. Cancer Lett 307: 174-181.

126. Liu QY, Tan BK (2002) Dietary fish oil and vitamin E enhance doxorubicin effects in P388 tumor-bearing mice. Lipids 37: 549-556.

127. Matsui H, Morishima I, Numaguchi Y, Toki Y, Okumura K, Hayakawa T (1999) Protective effects of carvedilol against doxorubicin-induced cardiomyopathy in rats. Life Sci 65: 1265-1274.

128. Machado V, Cabral A, Monteiro P, Goncalves L, Providencia LA (2008) Carvedilol as a protector against the cardiotoxicity induced by anthracyclines (doxorubicin). Rev Port Cardiol 27: 1277-1296.

129. Oliveira PJ, Rolo AP, Sardao VA, Monteiro P, Goncalves L, Providencia LA, Palmeira CM, Moreno AJ (2004) Advantages in the use of carvedilol versus propranolol for the protection of cardiac mitochondrial function. Rev Port Cardiol 23: 1291-1298.

130. Oliveira PJ, Santos DJ, Moreno AJ (2000) Carvedilol inhibits the exogenous NADH dehydrogenase in rat heart mitochondria. Arch Biochem Biophys 374: 279-285.

131. Nohl H (1987) Demonstration of the existence of an organo-specific NADH dehydrogenase in heart mitochondria. Eur J Biochem 169: 585-591.

132. Noguchi N, Nishino K, Niki E (2000) Antioxidant action of the antihypertensive drug, carvedilol, against lipid peroxidation. Biochem Pharmacol 59: 1069-1076.

133. Kalay N, Basar E, Ozdogru I, Er O, Cetinkaya Y, Dogan A, Inanc T, Oguzhan A, Eryol NK, Topsakal R, Ergin A (2006) Protective effects of carvedilol against anthracycline-induced cardiomyopathy. J Am Coll Cardiol 48: 2258-2262.

134. Hasinoff BB, Herman EH (2007) Dexrazoxane: how it works in cardiac and tumor cells. Is it a prodrug or is it a drug? Cardiovasc Toxicol 7: 140-144.

135. Swain SM, Whaley FS, Gerber MC, Weisberg S, York M, Spicer D, Jones SE, Wadler S, Desai A, Vogel C, Speyer J, Mittelman A, Reddy S, Pendergrass K, Velez-Garcia E, Ewer MS, Bianchine JR, Gams RA (1997) Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. J Clin Oncol 15: 1318-1332.

136. Kolaric K, Bradamante V, Cervek J, Cieslinska A, Cisarz-Filipcak E, Denisov LE, Donat D, Drosik K, Gershanovic M, Hudziec P, et al. (1995) A phase II trial of cardioprotection with Cardioxane (ICRF-187) in patients with advanced breast cancer receiving 5-fluorouracil, doxorubicin and cyclophosphamide. Oncology 52: 251-255.

137. Speyer JL, Green MD, Kramer E, Rey M, Sanger J, Ward C, Dubin N, Ferrans V, Stecy P, Zeleniuch-Jacquotte A, et al. (1988) Protective effect

of the bispiperazinedione ICRF-187 against doxorubicin-induced cardiac toxicity in women with advanced breast cancer. The New England journal of medicine 319: 745-752.

138. Voest EE, van Acker SA, van der Vijgh WJ, van Asbeck BS, Bast A (1994) Comparison of different iron chelators as protective agents against acute doxorubicin-induced cardiotoxicity. J Mol Cell Cardiol 26: 1179-1185.

139. Hasinoff BB, Patel D, Wu X (2003) The oral iron chelator ICL670A (deferasirox) does not protect myocytes against doxorubicin. Free Radic Biol Med 35: 1469-1479.

140. Kaiserova H, den Hartog GJ, Simunek T, Schroterova L, Kvasnickova E, Bast A (2006) Iron is not involved in oxidative stress-mediated cytotoxicity of doxorubicin and bleomycin. Br J Pharmacol 149: 920-930.

141. Liu XW, Chen ZG, Chua CC, Ma YS, Youngberg GA, Hamdy R, Chua BHL (2002) Melatonin as an effective protector against doxorubicin-induced cardiotoxicity. Am J Physiol Heart Circ Physiol 283: H254-H263.

142. Sehested M, Jensen PB, Sorensen BS, Holm B, Friche E, Demant EJF (1993) Antagonistic Effect of the Cardioprotector (+)-1,2-Bis(3,5-Dioxopiperazinyl-1-Yl)Propane (Icrf-187) on DNA Breaks and

Cytotoxicity Induced by the Topoisomerase-Ii Directed Drugs Daunorubicin and Etoposide (Vp-16). Biochem Pharmacol 46: 389-393.

143. Swain SM, Whaley FS, Gerber MC, Ewer MS, Bianchine JR, Gams RA (1997) Delayed administration of dexrazoxane provides cardioprotection for patients with advanced breast cancer treated with doxorubicin-containing therapy. J Clin Oncol 15: 1333-1340.

144. Jirkovsky E, Popelova O, Krivakova-Stankova P, Vavrova A, Hroch M, Haskova P, Brcakova-Dolezelova E, Micuda S, Adamcova M, Simunek T, Cervinkova Z, Gersl V, Sterba M (2012) Chronic anthracycline cardiotoxicity: molecular and functional analysis with focus on Nrf2 and mitochondrial biogenesis pathways. J Pharmacol Exp Ther.

145. Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN (1997) Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest 100: 1501-1506.

146. Xiong Y, Liu X, Lee CP, Chua BH, Ho YS (2006) Attenuation of doxorubicin-induced contractile and mitochondrial dysfunction in mouse heart by cellular glutathione peroxidase. Free Radic Biol Med 41: 46-55.

147. Kang YJ, Chen Y, Epstein PN (1996) Suppression of doxorubicin cardiotoxicity by overexpression of catalase in the heart of transgenic mice. J Biol Chem 271: 12610-12616.

148. Go YM, Jones DP (2011) Cysteine/cystine redox signaling in cardiovascular disease. Free Radic Biol Med 50: 495-509.

149. Jones DP (2008) Radical-free biology of oxidative stress. Am J Physiol Cell Physiol 295: C849-868.

150. Poljsak B, Milisav I (2012) The neglected significance of "antioxidative stress". Oxid Med Cell Longev 2012: 480895.

151. Chandran K, Aggarwal D, Migrino RQ, Joseph J, McAllister D, Konorev EA, Antholine WE, Zielonka J, Srinivasan S, Avadhani NG, Kalyanaraman B (2009) Doxorubicin inactivates myocardial cytochrome c oxidase in rats: cardioprotection by Mito-Q. Biophys J 96: 1388-1398.

152. Kotamraju S, Konorev EA, Joseph J, Kalyanaraman B (2000) Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. J Biol Chem 275: 33585-33592.

153. Zhang YW, Shi J, Li YJ, Wei L (2009) Cardiomyocyte death in doxorubicin-induced cardiotoxicity. Arch Immunol Ther Exp (Warsz) 57: 435-445.

154. Arola OJ, Saraste A, Pulkki K, Kallajoki M, Parvinen M, Voipio-Pulkki LM (2000) Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. Cancer research 60: 1789-1792.

155. Zhang YW, Shi J, Li YJ, Wei L (2009) Cardiomyocyte death in doxorubicin-induced cardiotoxicity. Arch Immunol Ther Exp 57: 435-445.

156. Andrieu-Abadie N, Jaffrezou JP, Hatem S, Laurent G, Levade T, Mercadier JJ (1999) L-carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. FASEB J 13: 1501-1510.

157. Yamaoka M, Yamaguchi S, Suzuki T, Okuyama M, Nitobe J, Nakamura N, Mitsui Y, Tomoike H (2000) Apoptosis in rat cardiac myocytes induced by Fas ligand: priming for Fas-mediated apoptosis with doxorubicin. J Mol Cell Cardiol 32: 881-889.

158. Zhu W, Soonpaa MH, Chen H, Shen W, Payne RM, Liechty EA, Caldwell RL, Shou W, Field LJ (2009) Acute doxorubicin cardiotoxicity is associated with p53-induced inhibition of the mammalian target of rapamycin pathway. Circulation 119: 99-106.

159. Yoshida M, Shiojima I, Ikeda H, Komuro I (2009) Chronic doxorubicin cardiotoxicity is mediated by oxidative DNA damage-ATM-p53-apoptosis pathway and attenuated by pitavastatin through the inhibition of Rac1 activity. J Mol Cell Cardiol 47: 698-705.

160. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y (1997) Induction of apoptosis by

ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275: 90-94.

161. Liu X, Chua CC, Gao J, Chen Z, Landy CL, Hamdy R, Chua BH (2004) Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice. Am J Physiol Heart Circ Physiol 286: H933-939.

162. Kim Y, Ma AG, Kitta K, Fitch SN, Ikeda T, Ihara Y, Simon AR, Evans T, Suzuki YJ (2003) Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. Mol Pharmacol 63: 368-377.

163. Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, Piantadosi CA (2007) The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. J Clin Invest 117: 3730-3741.

164. Fan GC, Zhou X, Wang X, Song G, Qian J, Nicolaou P, Chen G, Ren X, Kranias EG (2008) Heat shock protein 20 interacting with phosphorylated Akt reduces doxorubicin-triggered oxidative stress and cardiotoxicity. Circ Res 103: 1270-1279.

165. Ueno M, Kakinuma Y, Yuhki K, Murakoshi N, Iemitsu M, Miyauchi T, Yamaguchi I (2006) Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. J Pharmacol Sci 101: 151-158.

166. Green PS, Leeuwenburgh C (2002) Mitochondrial dysfunction is an early indicator of doxorubicin-induced apoptosis. Biochim Biophys Acta 1588: 94-101.

167. Kunisada K, Tone E, Negoro S, Nakaoka Y, Oshima Y, Osugi T, Funamoto M, Izumi M, Fujio Y, Hirota H, Yamauchi-Takihara K (2002) Bcl-xl reduces doxorubicin-induced myocardial damage but fails to control cardiac gene downregulation. Cardiovasc Res 53: 936-943.

168. Youn HJ, Kim HS, Jeon MH, Lee JH, Seo YJ, Lee YJ (2005) Induction of caspase-independent apoptosis in H9c2 cardiomyocytes by adriamycin treatment. Molecular and cellular biochemistry 270: 13-19.

169. Jang YM, Kendaiah S, Drew B, Phillips T, Selman C, Julian D, Leeuwenburgh C (2004) Doxorubicin treatment in vivo activates caspase-12 mediated cardiac apoptosis in both male and female rats. FEBS Lett 577: 483-490.

170. Velez JM, Miriyala S, Nithipongvanitch R, Noel T, Plabplueng CD, Oberley T, Jungsuwadee P, Van Remmen H, Vore M, St Clair DK (2011) p53 Regulates oxidative stress-mediated retrograde signaling: a novel mechanism for chemotherapy-induced cardiac injury. PLoS One 6: e18005.

171. Feridooni T, Hotchkiss A, Remley-Carr S, Saga Y, Pasumarthi KB (2011) Cardiomyocyte specific ablation of p53 is not sufficient to block

doxorubicin induced cardiac fibrosis and associated cytoskeletal changes. PLoS One 6: e22801.

172. Chua CC, Liu X, Gao J, Hamdy RC, Chua BH (2006) Multiple actions of pifithrin-alpha on doxorubicin-induced apoptosis in rat myoblastic H9c2 cells. Am J Physiol Heart Circ Physiol 290: H2606-2613.

173. Spallarossa P, Altieri P, Aloi C, Garibaldi S, Barisione C, Ghigliotti G, Fugazza G, Barsotti A, Brunelli C (2009) Doxorubicin induces senescence or apoptosis in rat neonatal cardiomyocytes by regulating the expression levels of the telomere binding factors 1 and 2. Am J Physiol Heart Circ Physiol 297: H2169-2181.

174. Maejima Y, Adachi S, Ito H, Hirao K, Isobe M (2008) Induction of premature senescence in cardiomyocytes by doxorubicin as a novel mechanism of myocardial damage. Aging Cell 7: 125-136.

175. Dimitrakis P, Romay-Ogando MI, Timolati F, Suter TM, Zuppinger C (2012) Effects of doxorubicin cancer therapy on autophagy and the ubiquitin-proteasome system in long-term cultured adult rat cardiomyocytes. Cell Tissue Res.

176. Lu L, Wu W, Yan J, Li X, Yu H, Yu X (2009) Adriamycin-induced autophagic cardiomyocyte death plays a pathogenic role in a rat model of heart failure. Int J Cardiol 134: 82-90.

177. Kaji A, Zhang Y, Nomura M, Bode AM, Ma WY, She QB, Dong Z (2003) Pifithrin-alpha promotes p53-mediated apoptosis in JB6 cells. Mol Carcinog 37: 138-148.

178. Eidenschink AB, Schroter G, Muller-Weihrich S, Stern H (2000) Myocardial high-energy phosphate metabolism is altered after treatment with anthracycline in childhood. Cardiol Young 10: 610-617.

179. Tokarska-Schlattner M, Zaugg M, da Silva R, Lucchinetti E, Schaub MC, Wallimann T, Schlattner U (2005) Acute toxicity of doxorubicin on isolated perfused heart: response of kinases regulating energy supply. Am J Physiol Heart Circ Physiol 289: H37-47.

180. Tokarska-Schlattner M, Wallimann T, Schlattner U (2002) Multiple interference of anthracyclines with mitochondrial creatine kinases: preferential damage of the cardiac isoenzyme and its implications for drug cardiotoxicity. Mol Pharmacol 61: 516-523.

181. Gratia S, Kay L, Potenza L, Seffouh A, Novel-Chate V, Schnebelen C, Sestili P, Schlattner U, Tokarska-Schlattner M (2012) Inhibition of AMPK signalling by doxorubicin: at the crossroads of the cardiac responses to energetic, oxidative, and genotoxic stress. Cardiovasc Res 95: 290-299.

182. Kockskamper J, Zima AV, Blatter LA (2005) Modulation of sarcoplasmic reticulum Ca2+ release by glycolysis in cat atrial myocytes. J Physiol 564: 697-714.

183. Sugiyama S, Satoh H, Nomura N, Terada H, Watanabe H, Hayashi H (2001) The importance of glycolytically-derived ATP for the Na+/H+ exchange activity in guinea pig ventricular myocytes. Molecular and cellular biochemistry 217: 153-161.

184. Abdel-aleem S, el-Merzabani MM, Sayed-Ahmed M, Taylor DA, Lowe JE (1997) Acute and chronic effects of adriamycin on fatty acid oxidation in isolated cardiac myocytes. J Mol Cell Cardiol 29: 789-797.

185. Brady LJ, Brady PS (1987) Hepatic and cardiac carnitine palmitoyltransferase activity. Effects of adriamycin and galactosamine. Biochemical pharmacology 36: 3419-3423.

186. Carvalho RA, Sousa RP, Cadete VJ, Lopaschuk GD, Palmeira CM, Bjork JA, Wallace KB (2010) Metabolic remodeling associated with subchronic doxorubicin cardiomyopathy. Toxicology 270: 92-98.

187. Hrelia S, Fiorentini D, Maraldi T, Angeloni C, Bordoni A, Biagi PL, Hakim G (2002) Doxorubicin induces early lipid peroxidation associated with changes in glucose transport in cultured cardiomyocytes. Biochim Biophys Acta 1567: 150-156.

188. Tokarska-Schlattner M, Lucchinetti E, Zaugg M, Kay L, Gratia S, Guzun R, Saks V, Schlattner U (2010) Early effects of doxorubicin in perfused heart: transcriptional profiling reveals inhibition of cellular stress response genes. Am J Physiol Regul Integr Comp Physiol 298: R1075-1088.

189. Andreadou I, Papaefthimiou M, Zira A, Constantinou M, Sigala F, Skaltsounis AL, Tsantili-Kakoulidou A, Iliodromitis EK, Kremastinos DT, Mikros E (2009) Metabonomic identification of novel biomarkers in doxorubicin cardiotoxicity and protective effect of the natural antioxidant oleuropein. NMR Biomed 22: 585-592.

190. Reuter SE, Evans AM (2012) Carnitine and acylcarnitines: pharmacokinetic, pharmacological and clinical aspects. Clin Pharmacokinet 51: 553-572.

191. Luo X, Reichetzer B, Trines J, Benson LN, Lehotay DC (1999) L-carnitine attenuates doxorubicin-induced lipid peroxidation in rats. Free Radic Biol Med 26: 1158-1165.

192. Mijares A, Lopez JR (2001) L-carnitine prevents increase in diastolic [CA2+] induced by doxorubicin in cardiac cells. Eur J Pharmacol 425: 117-120.

193. Sayed-Ahmed MM, Al-Shabanah OA, Hafez MM, Aleisa AM, Al-Rejaie SS (2010) Inhibition of gene expression of heart fatty acid binding protein and organic cation/carnitine transporter in doxorubicin cardiomyopathic rat model. Eur J Pharmacol 640: 143-149.

194. Yoon HR, Hong YM, Boriack RL, Bennett MJ (2003) Effect of L-carnitine supplementation on cardiac carnitine palmitoyltransferase activities and plasma carnitine concentrations in adriamycin-treated rats. Pediatr Res 53: 788-792.

195. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC (2010) Myocardial fatty acid metabolism in health and disease. Physiol Rev 90: 207-258.

196. Bar J, Davidi O, Goshen Y, Hod M, Yaniv I, Hirsch R (2003) Pregnancy outcome in women treated with doxorubicin for childhood cancer. Am J Obstet Gynecol 189: 853-857.

197. Davis LE, Brown CE (1988) Peripartum heart failure in a patient treated previously with doxorubicin. Obstet Gynecol 71: 506-508.

198. Katz A, Goldenberg I, Maoz C, Thaler M, Grossman E, Rosenthal T (1997) Peripartum cardiomyopathy occurring in a patient previously treated with doxorubicin. Am J Med Sci 314: 399-400.

199. Green DM, Fiorello A, Zevon MA, Hall B, Seigelstein N (1997) Birth defects and childhood cancer in offspring of survivors of childhood cancer. Arch Pediatr Adolesc Med 151: 379-383.

200. Ascensao A, Oliveira PJ, Magalhaes J (2011) Exercise as a beneficial adjunct therapy during Doxorubicin treatment-Role of mitochondria in cardioprotection. Int J Cardiol.

201. Hayward R, Lien CY, Jensen BT, Hydock DS, Schneider CM (2011) Exercise training mitigates anthracycline-induced chronic cardiotoxicity in a juvenile rat model. Pediatr Blood Cancer.

202. Black P, Gutjahr P, Stopfkuchen H (1998) Physical performance in long-term survivors of acute leukaemia in childhood. Eur J Pediatr 157: 464-467.

203. Steinherz L, Steinherz P (1991) Delayed cardiac toxicity from anthracycline therapy. Pediatrician 18: 49-52.

204. Chugun A, Temma K, Oyamada T, Suzuki N, Kamiya Y, Hara Y, Sasaki T, Kondo H, Akera T (2000) Doxorubicin-induced late cardiotoxicity: delayed impairment of Ca2+-handling mechanisms in the sarcoplasmic reticulum in the rat. Can J Physiol Pharmacol 78: 329-338.

205. Monti E, Prosperi E, Supino R, Bottiroli G (1995) Free radical-dependent DNA lesions are involved in the delayed cardiotoxicity induced by adriamycin in the rat. Anticancer Res 15: 193-197.

206. Sugiyama S, Yamada K, Hayakawa M, Ozawa T (1995) Approaches that mitigate doxorubicin-induced delayed adverse effects on mitochondrial function in rat hearts; liposome-encapsulated doxorubicin or combination therapy with antioxidant. Biochem Mol Biol Int 36: 1001-1007.

207. Villani F, Galimberti M, Monti E, Piccinini F, Poggi P, Lanza E, Rozza A, Favalli L (1991) Effect of flunarizine on the delayed cardiotoxicity of doxorubicin in rats. Pharmacol Res 23: 195-202.

208. Perletti G, Monti E, Paracchini L, Piccinini F (1989) Effect of trimetazidine on early and delayed doxorubicin myocardial toxicity. Arch Int Pharmacodyn Ther 302: 280-289.

209. Ramires PR, Ji LL (2001) Glutathione supplementation and training increases myocardial resistance to ischemia-reperfusion in vivo. Am J Physiol Heart Circ Physiol 281: H679-H688.

210. Venditti P, DiMeo S (1996) Antioxidants, tissue damage, and endurance in trained and untrained young male rats. Arch Biochem Biophys 331: 63-68.

211. Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, Shanely RA, Jessup J (1998) Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. Am J Physiol 275: R1468-1477.

212. Lumini JA, Magalhaes J, Oliveira PJ, Ascensao A (2008) Beneficial effects of exercise on muscle mitochondrial function in diabetes mellitus. Sports Med 38: 735-750.

213. Ascensao A, Magalhaes J, Soares J, Ferreira R, Neuparth M, Marques F, Oliveira J, Duarte J (2005) Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. Int J Cardiol 100: 451-460.

214. Wonders KY, Hydock DS, Schneider CM, Hayward R (2008) Acute exercise protects against doxorubicin cardiotoxicity. Integr Cancer Ther 7: 147-154.

215. Taylor RP, Harris MB, Starnes JW (1999) Acute exercise can improve cardioprotection without increasing heat shock protein content. Am J Physiol 276: H1098-1102.

216. Taylor RP, Olsen ME, Starnes JW (2007) Improved postischemic function following acute exercise is not mediated by nitric oxide synthase in the rat heart. Am J Physiol Heart Circ Physiol 292: H601-607.

217. Starnes JW, Taylor RP, Park Y (2003) Exercise improves postischemic function in aging hearts. Am J Physiol Heart Circ Physiol 285: H347-351.

218. Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS, Moore RL (2005) Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. J Physiol 564: 619-630.

219. Swain DP, Franklin BA (2002) Is there a threshold intensity for aerobic training in cardiac patients? Med Sci Sports Exerc 34: 1071-1075.

220. Ascensao A, Magalhaes J, Soares J, Oliveira J, Duarte JA (2003) Exercise and cardiac oxidative stress. Rev Port Cardiol 22: 651-678.

221. Pearson TA, Blair SN, Daniels SR, Eckel RH, Fair JM, Fortmann SP, Franklin BA, Goldstein LB, Greenland P, Grundy SM, Hong Y, Miller NH, Lauer RM, Ockene IS, Sacco RL, Sallis JF, Jr., Smith SC, Jr., Stone NJ, Taubert KA (2002) AHA Guidelines for Primary Prevention of Cardiovascular Disease and Stroke: 2002 Update: Consensus Panel

Guide to Comprehensive Risk Reduction for Adult Patients Without Coronary or Other Atherosclerotic Vascular Diseases. American Heart Association Science Advisory and Coordinating Committee. Circulation 106: 388-391.

222. Thompson PD, Buchner D, Pina IL, Balady GJ, Williams MA, Marcus BH, Berra K, Blair SN, Costa F, Franklin B, Fletcher GF, Gordon NF, Pate RR, Rodriguez BL, Yancey AK, Wenger NK, American Heart Association Council on Clinical Cardiology Subcommittee on Exercise R, Prevention, American Heart Association Council on Nutrition PA, Metabolism Subcommittee on Physical A (2003) Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease: a statement from the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity). Circulation 107: 3109-3116.

223. Chicco AJ, Schneider CM, Hayward R (2006) Exercise training attenuates acute doxorubicin-induced cardiac dysfunction. J Cardiovasc Pharmacol 47: 182-189.

224. Chen K, Xu X, Kobayashi S, Timm D, Jepperson T, Liang Q (2011) Caloric restriction mimetic 2-deoxyglucose antagonizes doxorubicin-induced cardiomyocyte death by multiple mechanisms. J Biol Chem 286: 21993-22006.

225. Mitra MS, Donthamsetty S, White B, Latendresse JR, Mehendale HM (2007) Mechanism of protection of moderately diet restricted rats against doxorubicin-induced acute cardiotoxicity. Toxicol Appl Pharmacol 225: 90-101.

226. Bardi E, Bobok I, A VO, Kappelmayer J, Kiss C (2007) Anthracycline antibiotics induce acute renal tubular toxicity in children with cancer. Pathol Oncol Res 13: 249-253.

227. Todorova VK, Kaufmann Y, Hennings L, Klimberg VS (2010) Oral glutamine protects against acute doxorubicin-induced cardiotoxicity of tumor-bearing rats. J Nutr 140: 44-48.

228. Todorova VK, Kaufmann Y, Hennings LJ, Klimberg VS (2010) Glutamine regulation of doxorubicin accumulation in hearts versus tumors in experimental rats. Cancer Chemother Pharmacol 66: 315-323.

229. Dresdale AR, Barr LH, Bonow RO, Mathisen DJ, Myers CE, Schwartz DE, d'Angelo T, Rosenberg SA (1982) Prospective randomized study of the role of N-acetyl cysteine in reversing doxorubicin-induced cardiomyopathy. Am J Clin Oncol 5: 657-663.

230. Zimmer HG (1998) The Isolated Perfused Heart and Its Pioneers. News Physiol Sci 13: 203-210.

231. Bell RM, Mocanu MM, Yellon DM (2011) Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. J Mol Cell Cardiol 50: 940-950.

232. Sutherland FJ, Hearse DJ (2000) The isolated blood and perfusion fluid perfused heart. Pharmacol Res 41: 613-627.

233. Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R (2007) Isolated heart perfusion according to Langendorff---still viable in the new millennium. J Pharmacol Toxicol Methods 55: 113-126.

234. Valentin JP, Hoffmann P, De Clerck F, Hammond TG, Hondeghem L (2004) Review of the predictive value of the Langendorff heart model (Screenit system) in assessing the proarrhythmic potential of drugs. J Pharmacol Toxicol Methods 49: 171-181.

235. Aasum E, Hafstad AD, Larsen TS (2003) Changes in substrate metabolism in isolated mouse hearts following ischemia-reperfusion. Mol Cell Biochem 249: 97-103.

236. Ashrafian H, Frenneaux MP, Opie LH (2007) Metabolic mechanisms in heart failure. Circulation 116: 434-448.

237. Korzeniewski B, Deschodt-Arsac V, Calmettes G, Gouspillou G, Franconi JM, Diolez P (2009) Effect of pyruvate, lactate and insulin on ATP supply and demand in unpaced perfused rat heart. Biochem J 423: 421-428.

238. Holden HM, Rayment I, Thoden JB (2003) Structure and function of enzymes of the Leloir pathway for galactose metabolism. J Biol Chem 278: 43885-43888.

239. Soares AF, Carvalho RA, Veiga FJ, Jones JG (2010) Effects of galactose on direct and indirect pathway estimates of hepatic glycogen synthesis. Metab Eng 12: 552-560.

240. Frey PA (1996) The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. FASEB J 10: 461-470.

241. Oppenheimer M, Valenciano AL, Sobrado P (2011) Biosynthesis of galactofuranose in kinetoplastids: novel therapeutic targets for treating leishmaniasis and chagas' disease. Enzyme Res 2011: 415976.

242. Gohil VM, Sheth SA, Nilsson R, Wojtovich AP, Lee JH, Perocchi F, Chen W, Clish CB, Ayata C, Brookes PS, Mootha VK (2010) Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis. Nat Biotechnol 28: 249-255.

243. Qu W, Oya S, Lieberman BP, Ploessl K, Wang L, Wise DR, Divgi CR, Chodosh LA, Thompson CB, Kung HF (2012) Preparation and characterization of L-[5-11C]-glutamine for metabolic imaging of tumors. J Nucl Med 53: 98-105.

244. Moreadith RW, Lehninger AL (1984) The pathways of glutamate and glutamine oxidation by tumor cell mitochondria. Role of mitochondrial NAD(P)+-dependent malic enzyme. J Biol Chem 259: 6215-6221.

245. Stanley WC, Recchia FA, Lopaschuk GD (2005) Myocardial substrate metabolism in the normal and failing heart. Physiol Rev 85: 1093-1129.

246. Schmidt MM, Dringen R (2009) Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. Front Neuroenergetics 1: 1.

247. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 278: 8516-8525.

248. Ricci JE, Waterhouse N, Green DR (2003) Mitochondrial functions during cell death, a complex (I-V) dilemma. Cell Death Differ 10: 488-492.

249. Gamble J, Lopaschuk GD (1994) Glycolysis and glucose oxidation during reperfusion of ischemic hearts from diabetic rats. Biochim Biophys Acta 1225: 191-199.

250. Beloukas AI, Magiorkinis E, Tsoumakas TL, Kosma AG, Diamantis A (2013) Milestones in the history of research on cardiac energy metabolism. Can J Cardiol 29: 1504-1511.

251. Dang CV, Le A, Gao P (2009) MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin Cancer Res 15: 6479-6483.

252. Sirover MA (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1432: 159-184.

253. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL, Cantley LC (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452: 230-233.

254. Strumilo S (2005) Short-term regulation of the mammalian pyruvate dehydrogenase complex. Acta Biochim Pol 52: 759-764.

255. Bonora M, Wieckowski MR, Chinopoulos C, Kepp O, Kroemer G, Galluzzi L, Pinton P (2014) Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition. Oncogene 0.

256. Liu Y, Chen XJ (2013) Adenine nucleotide translocase, mitochondrial stress, and degenerative cell death. Oxid Med Cell Longev 2013: 146860.

257. Paillard M, Tubbs E, Thiebaut PA, Gomez L, Fauconnier J, Da Silva CC, Teixeira G, Mewton N, Belaidi E, Durand A, Abrial M, Lacampagne A, Rieusset J, Ovize M (2013) Depressing mitochondria-reticulum interactions protects cardiomyocytes from lethal hypoxia-reoxygenation injury. Circulation 128: 1555-1565.

258. Felder TK, Soyal SM, Oberkofler H, Hahne P, Auer S, Weiss R, Gadermaier G, Miller K, Krempler F, Esterbauer H, Patsch W (2011) Characterization of novel peroxisome proliferator-activated receptor

gamma coactivator-1alpha (PGC-1alpha) isoform in human liver. J Biol Chem 286: 42923-42936.

259. Kang D, Kim SH, Hamasaki N (2007) Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. Mitochondrion 7: 39-44.

260. Nollen EA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115: 2809-2816.

261. Maiuri MC, Criollo A, Kroemer G (2010) Crosstalk between apoptosis and autophagy within the Beclin 1 interactome. EMBO J 29: 515-516.

262. Rogov V, Dotsch V, Johansen T, Kirkin V (2014) Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol Cell 53: 167-178.

263. Lippai M, Low P (2014) The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. Biomed Res Int 2014: 832704.

264. Louridas GE, Lourida KG (2012) Systems biology and biomechanical model of heart failure. Curr Cardiol Rev 8: 220-230.

265. Ventura-Clapier R, Garnier A, Veksler V, Joubert F (2011) Bioenergetics of the failing heart. Biochim Biophys Acta 1813: 1360-1372.

266. Dzeja PP, Redfield MM, Burnett JC, Terzic A (2000) Failing energetics in failing hearts. Curr Cardiol Rep 2: 212-217.

267. Ashrafian H (2002) Cardiac energetics in congestive heart failure. Circulation 105: e44-45.

268. Garnier A, Fortin D, Zoll J, N'Guessan B, Mettauer B, Lampert E, Veksler V, Ventura-Clapier R (2005) Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. FASEB J 19: 43-52.

269. Lushnikova EL, Klinnikova MG, Molodykh OP, Nepomnyashchikh LM (2004) Morphological manifestations of heart remodeling in anthracycline-induced dilated cardiomyopathy. Bull Exp Biol Med 138: 607-612.

270. Tacar O, Indumathy S, Tan ML, Baindur-Hudson S, Friedhuber AM, Dass CR (2014) Cardiomyocyte apoptosis vs autophagy with prolonged doxorubicin treatment: comparison with osteosarcoma cells. J Pharm Pharmacol.

271. Burkhoff D, Weiss RG, Schulman SP, Kalil-Filho R, Wannenburg T, Gerstenblith G (1991) Influence of metabolic substrate on rat heart function and metabolism at different coronary flows. Am J Physiol 261: H741-750.

272. Jones DP, Liang Y (2009) Measuring the poise of thiol/disulfide couples in vivo. Free Radic Biol Med 47: 1329-1338.

273. Gauthier LD, Greenstein JL, O'Rourke B, Winslow RL (2013) An integrated mitochondrial ROS production and scavenging model: implications for heart failure. Biophys J 105: 2832-2842.

274. Watanabe Y, Watanabe K, Kobayashi T, Saito Y, Fujioka D, Nakamura T, Obata JE, Kawabata K, Mishina H, Kugiyama K (2013) Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart. Cardiovasc Res 97: 282-292.

275. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP (1996) Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation 94: 2837-2842.

276. Bordoni A, Biagi P, Hrelia S (1999) The impairment of essential fatty acid metabolism as a key factor in doxorubicin-induced damage in cultured rat cardiomyocytes. Biochim Biophys Acta 1440: 100-106.

277. Dirks-Naylor AJ (2013) The role of autophagy in doxorubicin-induced cardiotoxicity. Life Sci.

278. Guo J, Guo Q, Fang H, Lei L, Zhang T, Zhao J, Peng S (2014) Cardioprotection against doxorubicin by metallothionein Is associated with preservation of mitochondrial biogenesis involving PGC-1alpha pathway. Eur J Pharmacol 737: 117-124.

279. Choi YS, Hong JM, Lim S, Ko KS, Pak YK (2006) Impaired coactivator activity of the Gly482 variant of peroxisome proliferator-activated

receptor gamma coactivator-1alpha (PGC-1alpha) on mitochondrial transcription factor A (Tfam) promoter. Biochem Biophys Res Commun 344: 708-712.

280. Kavazis AN, Smuder AJ, Powers SK (2014) Effects of short-term endurance exercise training on acute doxorubicin-induced FoxO transcription in cardiac and skeletal muscle. J Appl Physiol (1985) 117: 223-230.

281. Zhou P (2004) Determining protein half-lives. Methods Mol Biol 284: 67-77.

282. Varshavsky A (2005) Regulated protein degradation. Trends Biochem Sci 30: 283-286.

283. Zhou R, Vander Heiden MG, Rudin CM (2002) Genotoxic exposure is associated with alterations in glucose uptake and metabolism. Cancer Res 62: 3515-3520.

284. Kaukonen J, Juselius JK, Tiranti V, Kyttala A, Zeviani M, Comi GP, Keranen S, Peltonen L, Suomalainen A (2000) Role of adenine nucleotide translocator 1 in mtDNA maintenance. Science 289: 782-785.

285. Hansson A, Hance N, Dufour E, Rantanen A, Hultenby K, Clayton DA, Wibom R, Larsson NG (2004) A switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts. Proc Natl Acad Sci U S A 101: 3136-3141.

286. Graham JW, Williams TC, Morgan M, Fernie AR, Ratcliffe RG, Sweetlove LJ (2007) Glycolytic enzymes associate dynamically with mitochondria in response to respiratory demand and support substrate channeling. Plant Cell 19: 3723-3738.

287. Yuyun X, Jinjun Q, Minfang X, Jing Q, Juan X, Rui M, Li Z, Jing G (2013) Effects of Low Concentrations of Rotenone upon Mitohormesis in SH-SY5Y Cells. Dose Response 11: 270-280.

288. Higgins TJ, Bailey PJ (1983) The effects of cyanide and iodoacetate intoxication and ischaemia on enzyme release from the perfused rat heart. Biochim Biophys Acta 762: 67-75.

289. Cheng Y, Chen G, Hong L, Zhou L, Hu M, Li B, Huang J, Xia L, Li C (2013) How does hypoxia inducible factor-1alpha participate in enhancing the glycolysis activity in cervical cancer? Ann Diagn Pathol 17: 305-311.

290. Jeyaseelan R, Poizat C, Wu HY, Kedes L (1997) Molecular mechanisms of doxorubicin-induced cardiomyopathy. Selective suppression of Reiske iron-sulfur protein, ADP/ATP translocase, and phosphofructokinase genes is associated with ATP depletion in rat cardiomyocytes. J Biol Chem 272: 5828-5832.

291. Volkova M, Russell R, 3rd (2011) Anthracycline cardiotoxicity: prevalence, pathogenesis and treatment. Curr Cardiol Rev 7: 214-220.

292. Zhou S, Palmeira CM, Wallace KB (2001) Doxorubicin-induced persistent oxidative stress to cardiac myocytes. Toxicol Lett 121: 151-157.

293. Huss JM, Kelly DP (2005) Mitochondrial energy metabolism in heart failure: a question of balance. J Clin Invest 115: 547-555.

294. Leong HS, Brownsey RW, Kulpa JE, Allard MF (2003) Glycolysis and pyruvate oxidation in cardiac hypertrophy--why so unbalanced? Comp Biochem Physiol A Mol Integr Physiol 135: 499-513.

295. Pastorino JG, Hoek JB (2008) Regulation of hexokinase binding to VDAC. J Bioenerg Biomembr 40: 171-182.

296. Robey RB, Hay N (2005) Mitochondrial hexokinases: guardians of the mitochondria. Cell Cycle 4: 654-658.

297. Calmettes G, John SA, Weiss JN, Ribalet B (2013) Hexokinase-mitochondrial interactions regulate glucose metabolism differentially in adult and neonatal cardiac myocytes. J Gen Physiol 142: 425-436.

298. Patterson C, Ike C, Willis PWt, Stouffer GA, Willis MS (2007) The bitter end: the ubiquitin-proteasome system and cardiac dysfunction. Circulation 115: 1456-1463.

299. Gleyzer N, Scarpulla RC (2011) PGC-1-related coactivator (PRC), a sensor of metabolic stress, orchestrates a redox-sensitive program of inflammatory gene expression. J Biol Chem 286: 39715-39725.

300. Lee K, Qian DZ, Rey S, Wei H, Liu JO, Semenza GL (2009) Anthracycline chemotherapy inhibits HIF-1 transcriptional activity and tumor-

induced mobilization of circulating angiogenic cells. Proc Natl Acad Sci U S A 106: 2353-2358.

301. Yeung SJ, Pan J, Lee MH (2008) Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer. Cell Mol Life Sci 65: 3981-3999.

302. Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9: 425-434.