The effect of methylglyoxal in the regulation of connexin43 in cardiomyoblast cells

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Abbreviations

DM: Diabetes *Mellitus*

MGO: Methylglyoxal

AGEs: Advanced glycation end products

CVDs: Cardiovascular diseases

GJs: Gap junctions

Cx43: Connexin43

PTMs: Post-translational modifications

SUMO: Small ubiquitin-related modifier

DUBs: Deubiquitylating enzymes

SENPs: Sentrin-specific SUMO proteases

IP: Immunoprecipitation

DMEM: Dulbecco's modified Eagle's medium

FBS: Fetal bovine serum

PBS: Phosphate buffered saline

NEM: N-ethylmaleimide

WB: Western blot

SDS-PAGE: Sodium dudecyl sulfato polyacrylamide gel electrophoresis

PVDF: Polyvinylidene difluoride

TBS-T: Tris-buffered saline-Tween 20

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

IF: Immunofluorescence

PFA: Paraformaldehyde

BSA: Bovine serum albumin

DAPI: (4',6-diamidino-2-phenylindole)

Ct: Control

ODDD: Occulodentodigital dysplasia

Abstract

Cardiovascular diseases (CVDs) are one of the most frequent and fatal complications of Diabetes *Mellitus* (DM). Diabetes and hyperglycemia can lead to the accumulation of methylglyoxal (MGO), which has been associated to alterations of several proteins and impairment of cell and tissues including the heart. Protein modifications, such as SUMOylation and ubiquitination, are responsible for the regulation of several proteins, namely connexin43 (Cx43) which is the main component of gap junctions (GJs). GJs play an important role in heart function as they assure the propagation of the electrical impulse and myocardium synchronization. Hence GJs, and more specifically Cx43, need to be carefully regulated, among others, by SUMOylation and ubiquitination.

For the purpose of exploring the effects of hyperglycemia in post-translational modifications of Cx43 in cardiac cells, cardiomyoblasts H9c2 cells were treated with different concentrations of MGO to create a diabetes-like setting. The results, obtained by western blot and immunostaining, showed that MGO induced alterations on global levels of Cx43, SUMOylation and ubiquitination. Immunoprecipitations against Cx43 were also performed and it was observed that MGO can induce alterations on modifications of Cx43 by SUMO-2 and ubiquitin. This suggests that in our model of cardiac cells, a diabetic state can cause alterations on the regulation of Cx43, with subsequent impairment on cardiac intercellular communication and thus the normal heart function.

Therefore, this study aimed to shed light on the effects of a diabetes-like setting on the mechanisms involving regulation of Cx43 carried out by the post translational modifications,

SUMOylation and ubiquitination. In the future, these mechanisms may serve as potential therapeutic targets of diabetes.

Keywords: DIABETES; METHYLGLYOXAL; CONNEXIN43; UBIQUITINATION; SUMOYLATION.

Resumo

As doenças cardiovasculares são uma das complicações mais frequentes e fatais da Diabetes *Mellitus*. Diabetes e hiperglicémia podem levar à acumulação de metilglioxal, um composto capaz de se associar e modificar a função de várias proteínas e levando a uma desregulação celular e consequentemente a uma destruição de determinados tecidos como o tecido cardíaco. Estas modificações, tais como SUMOilação e ubiquitinação, são responsáveis pela regulação de várias proteínas, nomeadamente da conexina43 que é o principal constituinte dos canais intercelulares - *gap junctions*. As *gap junctions* assumem uma função importante no coração, pois asseguram a propagação do impulso eléctrico e sincronização do miocárdio. Posto isto, as *gap junctions* e mais especificamente a conexina43 necessitam de ser cuidadosamente reguladas, por várias modificações pós-tradução, nomeadamente por SUMOilação e ubiquitinação.

Com o intuito de explorar os efeitos da hiperglicémia nas modificações pós-tradução da conexina43 em células cardíacas, trataram-se cardiomioblastos H9c2 com diferentes concentrações de metilglioxal. O metilglioxal cria um ambiente semelhante ao diabético e os níveis de conexina43, ubiquitina, SUMO-1 e SUMO-2 foram analisados por western blot e imunofluorescência. Estes resultados evidenciaram que o metilglioxal induz alterações nos níveis de conexina43 e nos níveis totais de SUMOilação e ubiquitinação. Também se realizaram imunoprecipitações contra a conexina43 e observou-se que os tratamentos com metilglioxal podem provocar alterações na conexina43 modificada por SUMO-2 e ubiquitina. Estes resultados sugerem que, no nosso modelo de células cardíacas, a diabetes pode causar alterações na regulação da conexina43 por ubiquitinação e SUMOilação. Isto pode, por sua

vez, prejudicar a comunicação intercelular entre células cardíacas e consequentemente deteriorar a função cardíaca.

Assim, este estudo tem como objectivo esclarecer os efeitos de um ambiente semelhante ao da diabetes nos mecanismos que envolvem as modificações pós-tradução da conexina43 realizadas pela SUMOilação e ubiquitinação. No futuro, estas modificações da conexina43 poderão surgir como potenciais alvos terapêuticos da diabetes.

Palavras-chave: DIABETES; METILGLIOXAL; CONEXINA43; UBIQUITINAÇÃO; SUMOILAÇÃO.

Introduction

Diabetes *Mellitus* (DM) is a chronic metabolic disorder and it is currently one of the major health concerns worldwide due to its high prevalence, morbidity and mortality rates (1–3). One of its main features consists of high glucose levels (hyperglycemia) that can lead to an increase and accumulation of methylglyoxal (MGO) (3,4). MGO is a highly reactive dicarbonyl compound formed as a by-product of glycolysis which can cause oxidative stress, endothelial dysfunction and formation of advanced glycation end products (AGEs) by reacting with proteins, lipids and nucleic acids. AGEs can promote cellular dysfunction and are associated with diabetic complications (3–5). One of the most common and fatal complications of DM is the development of cardiovascular diseases (CVDs), hence the importance of understanding its mechanisms (6–9).

Intercellular communication, more specifically cell-to-cell direct communication, is an essential cardiac mechanism where two neighboring cells are connected by their plasmatic membrane, forming gap junctions (GJs) that allow the exchange of signaling molecules and propagation of electric conduction (10,11). Cardiac GJs can be formed by the extracellular docking of two hexameric hemichannels constituted by six subunits of a transmembrane protein named connexin43 (Cx43). Several members of the connexin family are expressed in the heart, however Cx43 is the most widely expressed and predominant in cardiac tissue (12–14). Cardiomyocytes, the main contractile cell unit of the heart, are characterized by their low mitotic activity that depends on a tight regulation of proteostasis. In addition, the unusual short half-life of Cx43, when compared to other transmembrane proteins, also relies on a precise and orchestrated proteostasis regulation. Therefore, it is imperative that Cx43 in

specific, and GJs in general, are precisely regulated to ensure a proper function of the heart (12,15,16).

Cx43 can be regulated by several post-translational modifications (PTMs), such as phosphorylation, glycosylation, S-nitrosylation, ubiquitination and SUMOylation. Ubiquitination and SUMOylation are defined as the covalent attachment of a ubiquitin protein and a small ubiquitin-like modifier (SUMO) to a target protein, respectively. This reaction involves a three-step enzymatic cascade that includes an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) (15–17). Four SUMO proteins have been identified in humans, SUMO-1, SUMO-2, SUMO-3 and SUMO-4 (18). Both processes are reversible through the action of deubiquitylating enzymes (DUBs) and sentrin-specific SUMO proteases (SENPs), for ubiquitination and SUMOylation pathways, respectively (15,19–21). Despite the similarity of both conjugation mechanisms, they present different cellular functions (22).

One of the major roles of ubiquitination is to target proteins for proteasome and lysosome degradation, whereas SUMOylation acts as a regulator and modulator of protein activity, function and localization (21,22). Some proteins, when modified by SUMO, can be resistant to proteasome degradation, suggesting a possible competition between SUMO and ubiquitin (22–25). Additionally, other substrates are regulated by a tight cooperation between ubiquitin and SUMO (17,22–25).

Recent studies have demonstrated that diabetes can lead to alterations on the levels of Cx43 (13,26). Others showed that MGO can have an impact on protein ubiquitination and that high glucose conditions can lead to alterations on protein modifications by SUMO (26–29). Based

on these evidences, we believe that, in addition to ubiquitination, SUMOylation might also regulate Cx43 in diabetes. Therefore, to address this question, H9c2 cardiomyoblasts cells were treated with MGO to mimic a hyperglycemic environment, creating a diabetes-like setting, and the levels of Cx43, ubiquitination and SUMOylation were analyzed (3,6). This approach might help us to understand some of the underlying mechanisms involving SUMOylation and ubiquitination of Cx43 on a diabetes-like condition, contributing to place Cx43 as a therapeutic target of diabetes.

Materials and methods

Cell culture and immunoprecipitations (IP)

The rat cardiomyoblast cell line H9c2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and 1% GlutaMax (1x). The cells were maintained at 37ºC in a humidified chamber with 5% CO_2 (30). On the day before the experiment, 1.5x10^6 cells were sown in a 100-mm plate for each experiment condition. On the next day, cells were treated or not with different MGO concentration for 1h, as indicated in each experiment. Then, cells were washed once in ice-cold phosphate buffered saline (PBS) 5ml/plate, lysed and scrapped in 500 μl of RIPA lysis buffer (150mM NaCl, 50mM Tris-Hcl, 1% NP-40 and 0.1% SDS, pH 7) supplemented with protease and phosphatase inhibitors and 20mM N-ethylmaleimide (NEM). Each cell lysate was sonicated for 10 seconds and then all samples were centrifugated at 13000 rpm for 10 minutes. After centrifugation, 1/50th of each lysate was saved for total protein extract analysis, and the remaining sample was used for the Cx43 immunoprecipitation (IP-Cx43) using 0,5 μg of antibody and 10 μl of protein G-Sepharose beads precondition. Immunoprecipitations were carried out for 2h, at 4ºC, rotating. Thereafter, G-Sepharose beads, were pellet down by centrifugation (5 minutes at 500xg, 4ºC) and washed 3 times with 1,5ml of PBS with 0.1% Tween20. In the end, immunoprecipitated proteins were eluted from G-sepharose beads with 20 μl of 2x loading buffer for western blot (WB) analysis as described below (31).

Western Blot

For western blot analysis, samples were denatured in 2x Laemmli buffer (5% SDS, 25% glycerol, 150mM Tris-pH 6,8; 0,01% bromophenol blue; 10% β− mercaptoethanol) boiled for

5 minutes at 95ºC, separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), and transferred to PVDF (polyvinylidene difluoride) membranes. The membranes were blocked in a solution of 5% non-fat milk in 1x of Tris-buffered saline-Tween 20 (TBS-T) (20mM Tris, 150mM NaCl, 0,2% Tween 20, pH 7.6) and membranes were probed with the appropriate primary antibodies and the respective secondary-HPR (horseradish peroxidase) conjugated secondary antibodies, as follows: anti-Cx43 (goat polyclonal), anti-SUMO-1 (mouse monoclonal), anti-SUMO2 (mouse monoclonal), anti-ubiquitin P4D1 (mouse monoclonal) and anti-Calnexin (goat polyclonal). All primary antibodies were incubated overnight using an 1/1000 dilution, and the secondary antibodies were incubated for 45 minutes at 1:10000 dilution. Proteins were analyzed and visualized by chemiluminescence using the VersaDoc system from BioRad (32).

MTT cell viability assay

For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, 5x10^3 of H9c2 cells per well were sown into a 96-well plate. On the day of the experiment cells were treated or not with the indicated concentrations of MGO, for 1h, for cell viability analysis. After treatments, cells were carefully washed twice with 0,2 ml of PBS at room temperature and then incubated with 0,5mg/ml of MTT reagent diluted in DMEM for 2 hours at 37ºC in a cell culture incubator. Then, the supernatant was carefully removed and the precipitated dye was dissolved in 0,3ml of a solution of 0,04M HCl dissolved in isopropanol and quantified at a wavelength of 570nm, with wavelength correction at 620nm, using Biotek Synergy HT spectrophotometer (28).

Immunofluorescence staining (IF)

For immunofluorescence assays, H9c2 cells were cultured in a 6-well plate, on the day before with 0,3x10^6 cells/well. On the next day, cells were treated or not (control) with 1, 2, 3, 4, 5mM MGO for 1h. Then, cells were washed twice with 1ml/well of ice-cold PBS and fixed in 4% of paraformaldehyde (PFA) for 10 minutes, followed by three washes with PBS. Subsequently, cells were permeabilized with 0.2% v/v Triton x-100 in PBS, for 10 minutes, and blocked with 2.5% bovine serum albumin (BSA) for 20 minutes. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) and SUMO-2 modified proteins were analyzed using the 8A2 monoclonal antibody, at 1:100 dilution, further conjugated to a fluorescent Alexa 594 anti-mouse with a 1:200 dilution. All solutions were made in 0.25% w/v BSA containing 0.02% sodium azide in PBS. For controls (Ct), primary antibodies were omitted. The images were collected by fluorescence microscopy using a Zeiss Axio HXP IRE 2 and analyzed by Image J software (32).

Results

Accumulation of methylglyoxal, is considered a potential link between hyperglycemia and diabetes complications (33). MGO can accumulate in different tissues leading to the formation of certain byproducts which have been implicated in cell and tissue impairment underlying several maladies, including cardiovascular diseases (4). These impairments, in many cases related to alterations in several metabolic pathways, are accompanied by a variety of post-translational protein modifications that in turn can reflect diabetic glucotoxicity (34). MGO has been implicated in modifications of proteins through an ubiquitination-mediated mechanism as well as through SUMOylation (29,33). Moreover, alterations in Cx43 have been described in diabetic complications (26,35).

In addition to SUMOylation and ubiquitination, other modifications such as nitration, cysteine S-nitrosylation, acetylation, ADP-ribosylation, have also been linked to diabetic and hyperglycemia conditions (34). Studies attribute these PTMs to the activation of pathways, such as the polyol and the ADP-ribosylation pathways that mediate the diabetic metabolic imbalance involving oxidative and/or carbon stress (34). Exploring the mechanisms of these modifications in a diabetes-like setting may improve the understanding of this disease and its associated complications.

Previous results obtained by our research group showed that hyperglycemia and accumulated MGO is associated with Cx43 ubiquitination-induced degradation in H9c2 cells (36). Based on these evidences, the current work aims to determine if MGO could also have an impact on Cx43 SUMOylation. With this purpose, we used the same experimental conditions previously described to investigate the effect of MGO in Cx43 ubiquitination in H9c2 cells. Therefore,

cells were treated with 3mM MGO, for 1h, after which the global levels of SUMOylation and ubiquitination were evaluated by western blot analysis.

The results in Figure 1A (global modified proteins) show that 3mM of MGO induces a global increase of proteins modified by SUMO-1, SUMO-2 and ubiquitin. Moreover the total amount of Cx43 (Figure 1A) levels decreased after incubation with MGO, when compared to cells maintained in control conditions. Calnexin levels were used as loading control.

Subsequently, to evaluate the impact of these MGO treatments on Cx43 SUMOylation, the same cell lysates were immunoprecipitated using an antibody against Cx43, after which the levels of modified protein were assessed by western blot (Figure 1B - modified Cx43). These results indicate that, at basal conditions (no MGO treatment) Cx43 is modified by SUMO-2 and ubiquitin. However, these modifications decrease after the treatment of MGO, most probably due to MGO induced Cx43 ubiquitination and degradation, as previously reported and demonstrated in this laboratory (36). Regarding to the modification of Cx43 by SUMO-1 we could not conclude if this modification is affected by MGO treatment.

Since the experimental conditions used above were those previously reported for MGO-induced Cx43 ubiquitination, we perfomed a dose response study, to evaluate the impact of increasing concentrations of MGO on Cx43 SUMOylation. For that H9c2 cells were incubated with MGO, for 1h, with different concentrations of MGO, from 1 to 5mM. Before proceeding with immunoprecipitation assays, a MTT assay was performed to address if MGO concentrations were affecting cell viability. As it can be observed in Figure 2A, no significant differences between untreated and treated conditions affected cell viability.

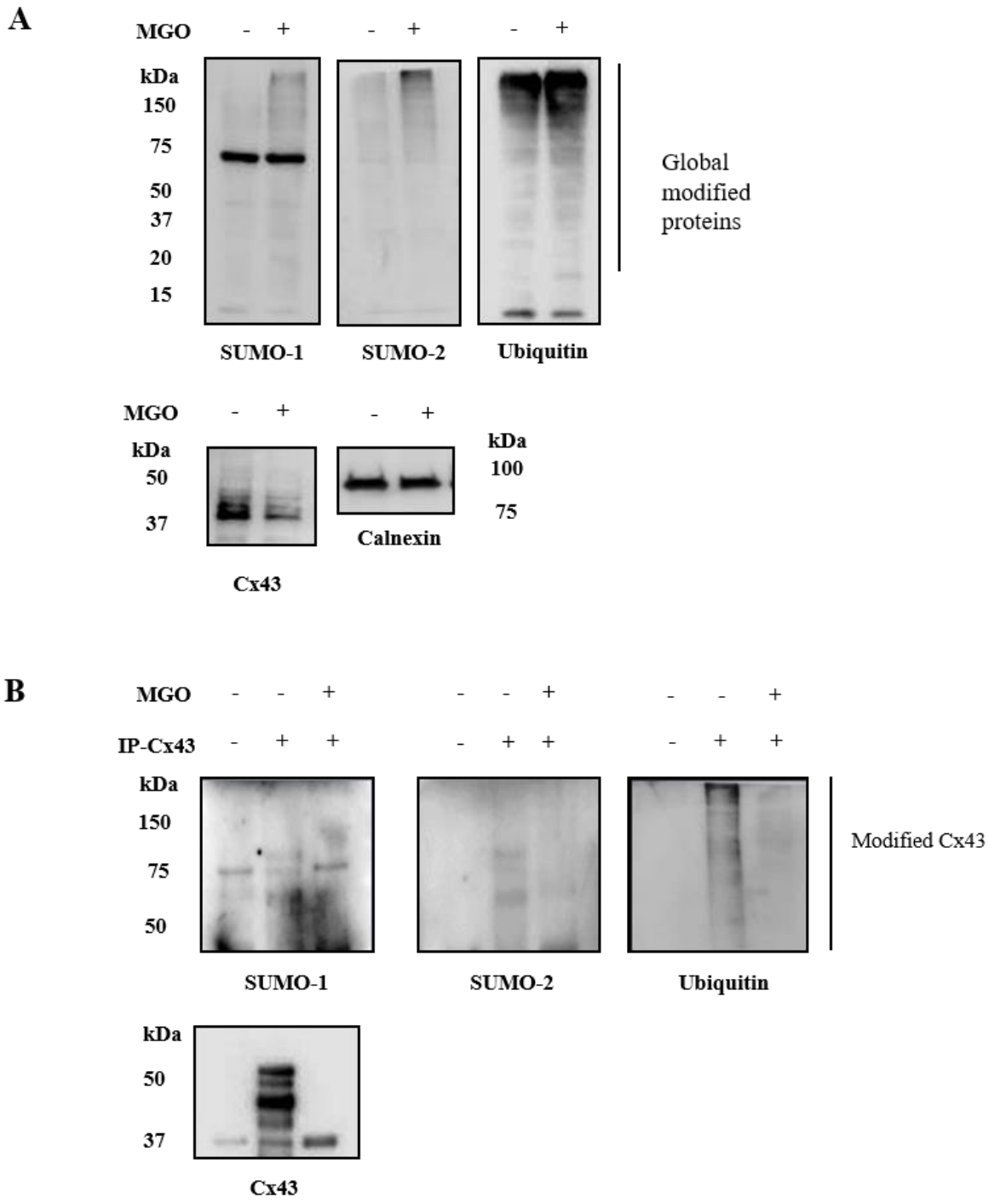


Figure 1. MGO induces alterations in Cx43, SUMOylation and ubiquitination levels.

H9c2 cells were treated with 0 (-) or 3mM (+) of MGO for 1h. (A) WB was performed to determine whether MGO induces alterations in global levels the following proteins: SUMO-1, SUMO-2, ubiquitin and Cx43. The loading control used was calnexin. (B) Immunoprecipitations of Cx43 (IP-Cx43) were performed to verify if MGO treatment induces changes in Cx43 modification by SUMO-1, SUMO-2 and ubiquitin.

With respect to SUMOylation levels, as it is shown on Figure 2B initial concentrations of MGO (1 to 3mM) led to a gradual increase of global SUMO-2 conjugates. However, higher concentrations of MGO such as 4 and 5mM causes a decrease of global SUMO-2 conjugates. Concerning to protein ubiquitination, MGO also seems to induce an increase of total ubiquitinated substrates at lower concentrations (1 to 3mM) with a slight reduction in higher concentrations (4 and 5mM). The crescent concentrations of MGO also lead to a reduction of the total levels of Cx43. To further investigate the effect of these MGO concentrations on Cx43 SUMOylation, immunoprecipitations against Cx43 and analysis by western blot against SUMO-2 (Figure 2C) were carried out. These results indicate that 1mM concentration of MGO induces a modest increase of SUMO-2 modified Cx43 when compared to the control (no MGO). On the other hand, concentrations above 2mM lead to a decrease of SUMOylated Cx43.

To compare the differential effect of MGO on Cx43 SUMOylation and ubiquitination, in addition to SUMO, we evaluated the amount of ubiquitin attached to Cx43, in our experimental conditions. According to results from Figure 2B the levels of total ubiquitination are generally higher than total SUMOylation. However, SUMOylation levels slightly increase when cells are treated with lower MGO concentrations (1mM and 2mM) and then decrease at higher concentrations (3-5mM). Concerning the levels of Cx43 ubiquitination (Figure 2C), there is a small decrease when MGO concentrations were 1mM and 2mM, compared with untreated condition (0mM). At 3mM, though, it can be observed a small increase in Cx43 ubiquitinated, with subsequent decrease at 4mM and 5mM conditions. Moreover, the levels of Cx43, gradually decrease with higher concentrations of MGO. In this experiment, we decided to focus only in Cx43 modification by SUMO-2 and not to include SUMO-1 since the previous results presented in Figure1 were not conclusive.

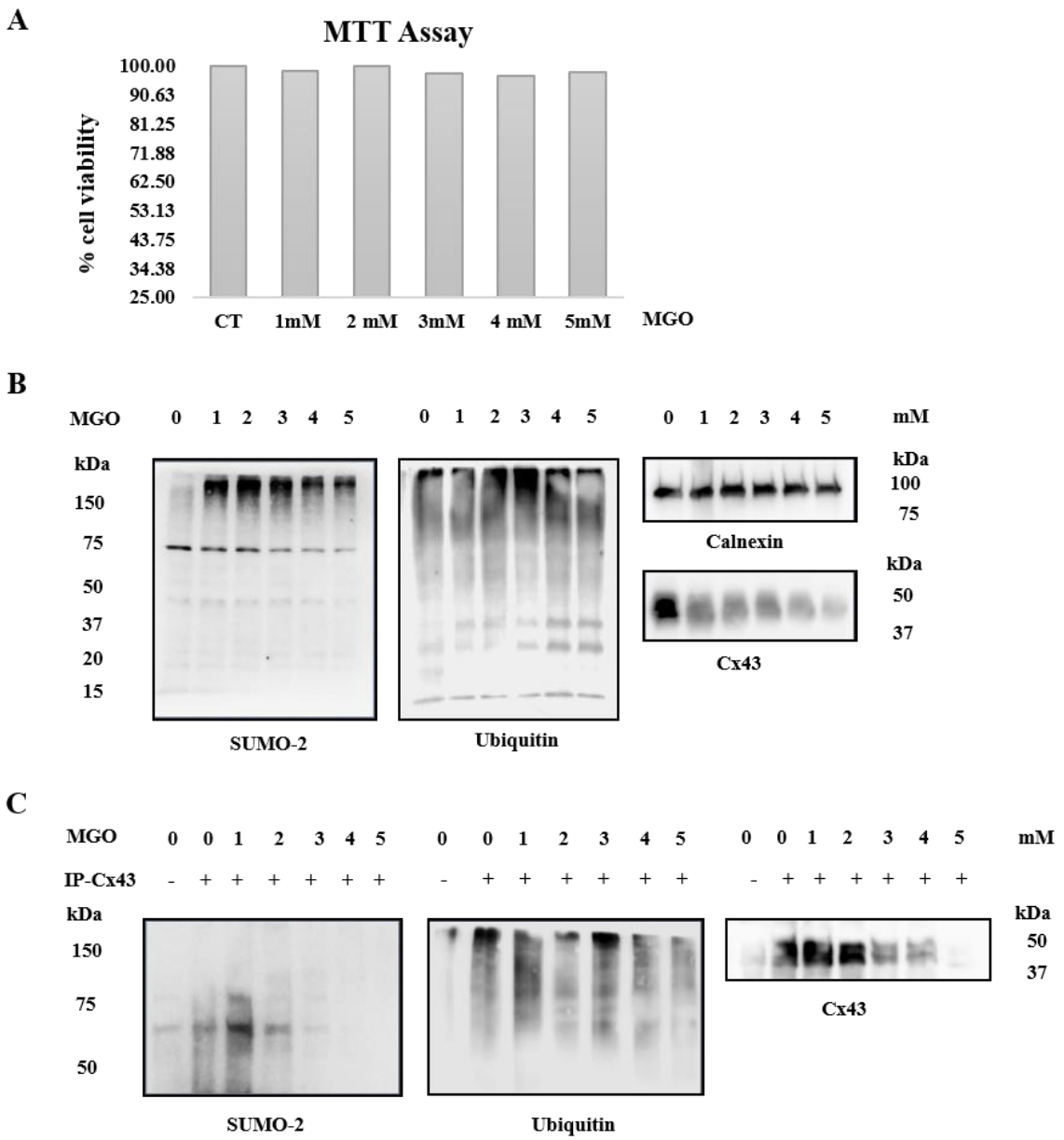
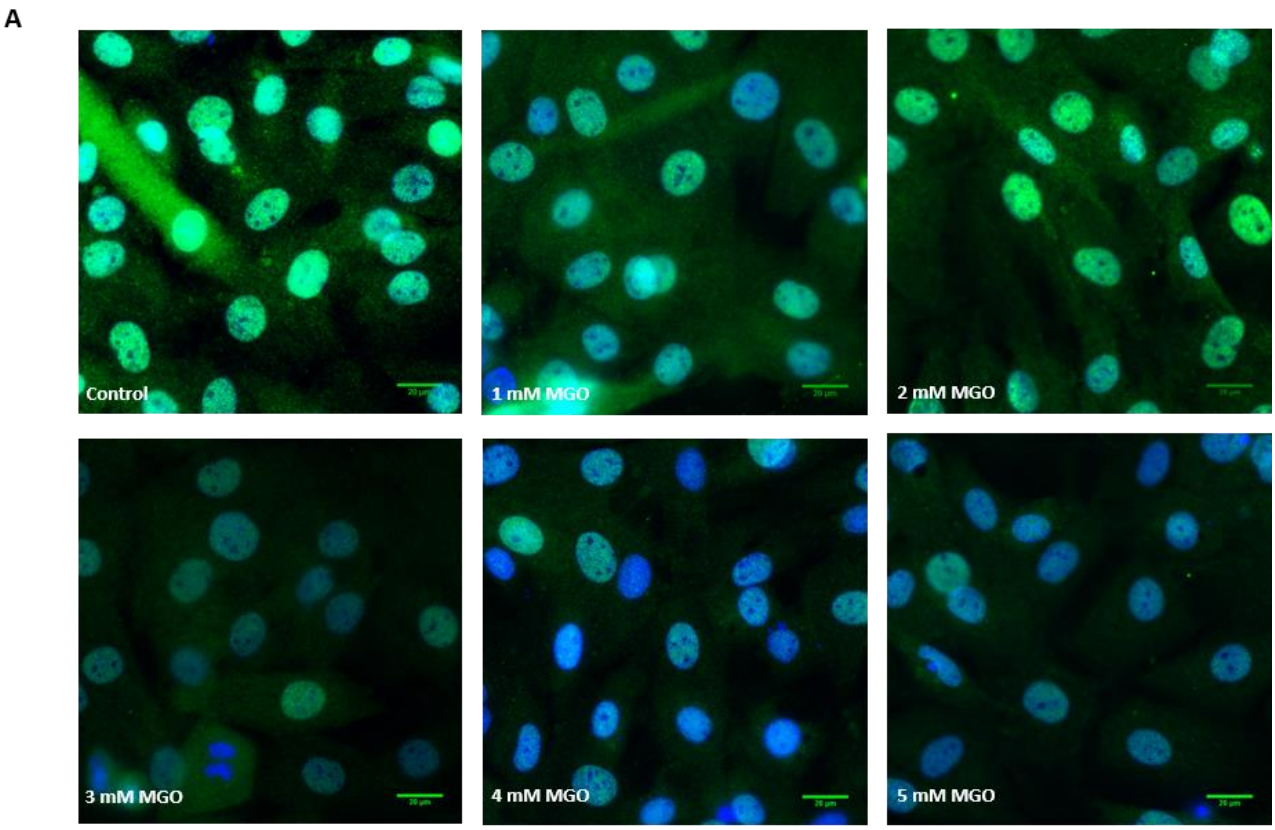


Figure 2. MGO induces alterations in SUMOylation, ubiquitination and Cx43 levels.

H9c2 cells were treated with different concentrations of MGO (0, 1, 2, 3, 4 and 5mM) for 1 hour. (A) MTT assay was carried out to verify the impact of MGO on cell viability. The graph shows the percentage of cell viability for control (Ct) cells and cells treated with different concentrations of MGO. (B) WB were performed in order to study the effects of MGO in the following proteins: Cx43, SUMO-2 and ubiquitin. The loading control used was calnexin. (C) Cx43 was immunoprecipitated (IP-Cx43) after which the levels of Cx43 modified by ubiquitin and SUMO-2 were analized. These IPs were performed to verify if MGO induced alterations in the levels of Cx43 modified by SUMO-2 and ubiquitin.



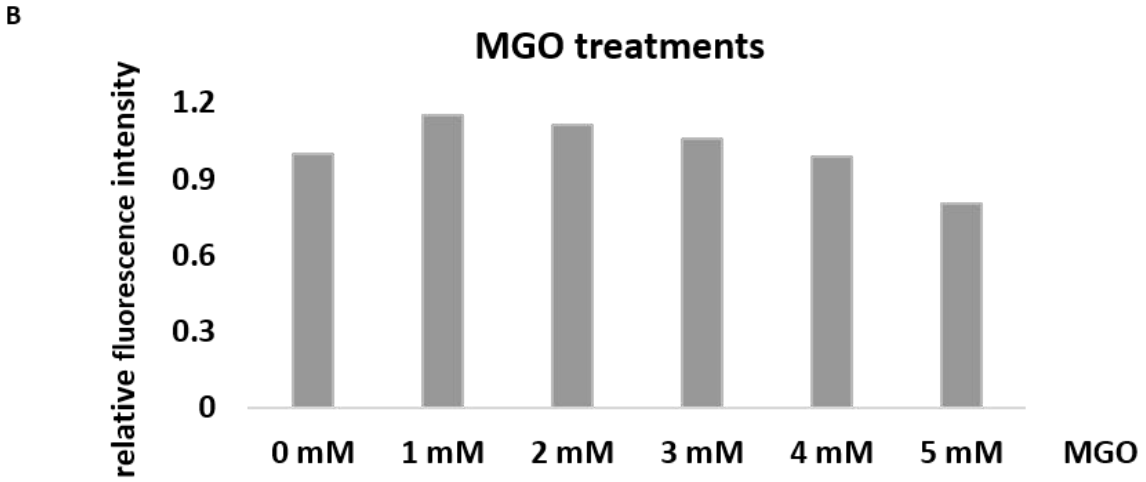


Figure 3. MGO leads to alterations in global SUMOylation.

H9c2 cells were treated with 0 (control), 1, 2, 3, 4, 5mM of MGO for 1 hour. (A) Immunostaining for SUMO-2 (green) in H9c2 cells treated with different concentrations of MGO. Nuclei were stained with DAPI (blue). (B) Graph indicating the relative fluorescence intensity for each MGO concentration used.

To further confirm the MGO-induced increase of SUMOylation, we used immunofluorescence assays with antibodies against SUMO2, in H9c2 cells treated with different concentrations of MGO, for 1h. As it can be observed in Figure 3, variation profile of total SUMOylation is similar to that observed in WB analysis, where low concentrations of MGO (1-2mM) increase slightly the levels of total SUMOylated proteins. On the contrary, higher MGO concentrations (3-5mM) conduct to a significant reduction of total SUMOylation.

Discussion

The presence of GJs between cardiac cells is necessary to ensure the correct propagation of the electrical impulse and thus the synchronization of the electrical activity of the myocardium (37). Given that most of cardiac GJs are constituted by connexin43, this protein needs to be carefully regulated by proteolytic systems, in order to guarantee the proper function of the heart (35).

Proteolysis is an essential mechanism in the maintenance of protein homeostasis and thus cell survival (15). It is particularly important in cells with a low mitotic activity, since the chronic accumulation of damaged proteins may impair the normal function of the cell (14,15,38). Cardiac cells are a good example of cells that rely on these mechanisms to ensure protein quality control (15). The three most important proteolytic systems in the heart are ubiquitin-proteasome system, autophagy and the calpain system (17). These proteolytic systems are regulated by many molecular mechanisms, such as post-translational modifications like phosphorylation, ubiquitylation and SUMOylation (17). As many other proteins, Cx43 can be regulated by these PTMs (16,39).

Connexins are widely distributed through the body, and for this reason its alterations have been associated with a large spectrum of diseases such as X-linked Charcot-Marie-Tooth syndrome, skin disorders, cataracts, deafness and occulodentodigital dysplasia (ODDD) (12,40,41). Previous studies have also linked Cx43 to diabetes (26).

Such studies suggest that diabetes may cause different alterations of Cx43 levels depending on the tissue or localization. For instance, it has been demonstrated that Cx43 can be

downregulated and redistributed in diabetic retinopathy and diabetic nephropathy (26). Moreover, a study performed on streptozotocin-induced diabetes in the heart of rats showed an increase of phosphorylated Cx43 in the ventricles that can be associated with an increase on its proteolytic degradation (35). Another study reported that high glucose levels on bovine aortic smooth muscle cell cultures can inhibit the activity of GJs (42). However, other studies demonstrated that high glucose treatments in collecting duct cells, diabetic foot ulcers and diabetic heart atria, can promote an upregulation of Cx43 (26).

This study demonstrates that MGO treatments can lead to a decrease of Cx43 protein levels registered on H9c2 cells. Based on this it can be inferred that diabetes can be responsible for a decrease of GJs and thus an impairment in intercellular communication. Clinically, a decrease in cardiac GJs is associated with a reduction of the conduction velocity and disruption of impulse propagation that may originate cardiac arrhythmias (43,44). Recent data indicates that the decrease of Cx43 can result from PKC (Protein kinase C) activation that causes hyperphosphorylation of Cx43 and subsequent targeting for ubiquitination and degradation, culminating in GJs reduction (43). The present study suggests that other PTMs, besides phosphorylation and ubiquitination, such as SUMOylation may be implicated in Cx43 modulation in diabetes.

Other researchers have already investigated the effects of treatment with MGO in ubiquitination and SUMOylation. Some have shown that treating cells with MGO leads to an increase in ubiquitin conjugated and to a decrease in free ubiquitin and 20S proteasome activity (27,28). Others have described an upregulation of proteasomal activity in an acute phase of diabetes and downregulation in a chronic phase (27). Furthermore, MGO has been previously implicated in affecting proteins regulation through a ubiquitination-mediated

mechanism (33). In relation to SUMOylation, previous research studies have shown alterations on SUMO substrates such as IκBα (I kappa B alpha) and ER (extracellular signal-regulated kinases) that, under high glucose conditions, are more intensively SUMOylated. These observations suggest that SUMOylation can be an important regulator of endothelial dysfunction and inflammation-related proteins (29).

The present work depicts early observations that MGO treatments can induce changes on global levels of ubiquitination and SUMOylation. Concerning to Cx43, MGO treatments can also induce alterations on the levels of Cx43 itself and in post-translational modifications of Cx43 by ubiquitination and SUMOylation. Per the results of Figure 1, it was observed that when H9c2 cells are treated with 3mM MGO for one hour, the levels of Cx43 decreased, most probably due to induced ubiquitination and proteolytic degradation. In order to assess if different MGO concentrations can have different impacts on Cx43, an additional experiment was conducted (Figure 2). These results indicate that there is an increase in Cx43 SUMOylated when cells were treated with 1mM MGO for one hour followed by a decrease with higher concentrations (2-5mM) of MGO (Figure 2B).

The levels of ubiquitination of Cx43 suggest that Cx43 degradation may occur quickly thus impeding its clear observation. Additional experiments using intermediate concentrations, between 1mM and 2mM of MGO for 1 hour, as well as analysis involving lysosome and proteasome inhibitors, would be helpful to better understand the relation between ubiquitination and SUMOylation of Cx43. These modifications have been described as competitors or cooperators, depending on the substrate and cellular conditions (22–25). In order to further explore this hypothesis, an experiment testing the use of ubiquitination inhibitors could potentially show an increase in the levels of Cx43 SUMOylated and suggest a

competitive interaction between these two mechanisms. Another interesting approach would be to compare the results of different treatment times and establish a correlation with acute and chronic diabetes. Immunostaining of SUMO-2 (Figure 3) was performed to complement the results on Figure 2 and both evidence the same pattern of SUMOylation. Co-localization of SUMO-2 with Cx43 would be an interesting experiment to visualize the distribution of both proteins.

Cx43 has already been studied and proposed as a therapeutic target in conditions such as diabetic wound healing and arrhythmias (14,45). It was also reported that using GJs inhibitors would reduce the infarct size (14). In this study it is suggested that diabetes leads to alterations in Cx43 modified by ubiquitin and SUMO-2 which may impair the intercellular communication in the heart. Further research is needed to understand whether these observations are beneficial or prejudicial to the cardiac cells in order to use Cx43 and its post-translational modifications as a potential therapeutic target of diabetes.

Conclusion

This preliminary study explored the impact of MGO - a mimicked situation of DM – in the regulation of Cx43 H9c2 cells.

In our model of cardiac cells, it was observed that MGO can lead to alterations on global levels of Cx43, ubiquitination and SUMOylation. More specifically, MGO treatments induced changes in Cx43 ubiquitination and SUMOylation. Therefore, in addition to ubiquitination, this work reinforces the role of Cx43 as a SUMOylation substrate protein. It is hypothesized that the SUMO system is involved in the regulation of Cx43 gap junctions when cells are placed on a diabetic-like state.

The finding that Cx43 is modified by SUMO adds another level to the complexity of gap junction regulation, with important implications for future studies on the deregulation of gap junctions in human pathologies as well as identifying new potential therapeutic targets (39).

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