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Street heroin induces mitochondrial dysfunction and apoptosis in rat cortical neurons

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Abstract

Cortical function has been suggested to be highly compromised by repeated heroin self-administration. We have previously shown that *street* heroin induces apoptosis in neuronal-like PC12 cells. Thus, we analysed the apoptotic pathways involved in *street* heroin neurotoxicity using primary cultures of rat cortical neurons. Our *street* heroin sample was shown to be mainly composed by heroin, 6-monoacetylmorphine and morphine. Exposure of cortical neurons to *street* heroin induced a slight decrease in metabolic viability, without loss of neuronal integrity. Early activation of caspases involved in the mitochondrial apoptotic pathway was observed, culminating in caspase 3 activation, Poly-ADP Ribose Polymerase (PARP) cleavage and DNA fragmentation. Apoptotic morphology was completely prevented by the non-selective caspase inhibitor z-VAD-fmk, indicating an important role for caspases in neurodegeneration induced by *street* heroin. Ionotropic glutamate receptors, opioid receptors and oxidative stress were not involved in caspase 3 activation. Interestingly, *street* heroin cytotoxicity was shown to be independent of a functional mitochondrial respiratory chain, as determined using NT-2 rho^0 cells. Nonetheless, in *street* heroin-treated cortical neurons, cytochrome *c* was released, accompanied by a decrease in mitochondrial potential and Bcl-2/Bax. Pure heroin hydrochloride similarly decreased metabolic viability but only slightly activated caspase 3. Altogether, our data suggest an important role for mitochondria in mediating *street* heroin neurotoxic effects.

Keywords: apoptosis, cell death, heroin, mitochondrial dysfunction.

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Drug addiction seriously affects public health worldwide. Amongst the most abused illicit drugs, opioids, such as heroin cause some of the most severe effects. The Annual Report of the European Monitoring Centre for Drugs and Drug Addiction (2006) refers that opiates remain the principal drugs for which people seek treatment associated with illicit substances in Europe.

Heroin abuse, in particular, has been associated with loss of impulse control (Pau *et al.* 2002), a function of the frontal cortex associated with drug dependence. Furthermore, down-regulation of mu-opioid receptors was reported to occur in the prefrontal cortex in human heroin addicts, as detected by post-mortem analysis (Ferrer-Alcon *et al.* 2004).

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Abbreviations used: 6-MAM, 6-monoacetyl morphine; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide; d-AP-5, d-2-amino-5-phosphonopentanoic acid; GSH-EE, glutathione ethyl ester; LDH, lactate dehydrogenase; MDM2, mouse double minute-2; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzoquinoxaline-7-sulfonamide; PMSF, phenylmethylsulfonyl fluoride; Rh123, rhodamine 123; ROS, reactive oxygen species; SBTI, soybean trypsin inhibitor.

Street heroin may contain different quantities of heroin and other components depending on its origin and on the method of illicit synthesis. Heroin is a semi-synthetic drug, obtained from acetylation of morphine. Heroin possesses little or no opioid activity (White and Irvine 1999) but its metabolism, which may occur *in vivo* and *in vitro* (Hutchinson and Somogyi 2002), generates 6-monoacetylmorphine (6-MAM) and morphine, two mu-opioid receptor agonists (White and Irvine 1999). *Street* heroin is illegally synthesized from morphine purified from opium extracts. Opium contains about 40 alkaloids that make up 10–20% of total opium substances. The most abundant opium alkaloids are morphine (8–17%), codeine (0.7–5%), thebaine (0.1–1.5%), papaverine (0.5–1.5%) and noscapine (narcotine 1–10%) (Schiff 2002). Upon illicit purification, morphine is often contaminated with other alkaloids, which may also suffer synthetic acetylation during heroin manufacture. Depending on the purification procedure, *street* heroin may contain some impurities (Moore *et al.* 1984), namely morphine and 6-MAM (heroin metabolites), codeine and acetylcodeine (Soine 1986; for review). The heroin content of seized samples may also contain various inert diluents (starch, lactose, fructose, sucrose, mannitol and powdered milk) and active adulterants (caffeine, paracetamol, strychnine, acetylsalicylic acid, barbiturates, quinine and amphetamines) (Chiarotti *et al.* 1991; Sharma *et al.* 2005).

Apoptotic cell death appears to be involved in the loss of neuronal function induced by opioids. We have previously shown that *street* heroin induces apoptosis in neuronal-like PC12 cells (Oliveira *et al.* 2002, 2003). Furthermore, morphine was described to induce apoptosis in neurons (Mao *et al.* 2002) and microglia (Hu *et al.* 2002).

Apoptosis may be initiated by the activation of the extrinsic and/or the intrinsic pathways. The extrinsic pathway is activated by death receptors in the cell membrane that further activate the initiator caspases 8 or 10 (Chen and Wang 2002) and possibly caspase 2 (Zhivotovsky and Orrenius 2005). The intrinsic pathway is initiated by an increase in mitochondrial membrane permeability, regulated by pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family, and associated with the loss of mitochondrial potential ($\Delta\Psi_m$) and the release of mitochondrial proteins to the cytosol (Scorrano and Korsmeyer 2003). Among these proteins, the most studied is cytochrome *c*, which induces the formation of the apoptosome and the consequent activation of caspase 9. Caspase 2 may also trigger the intrinsic pathway (Enoksson *et al.* 2004). Initiator caspases activate effector caspases (such as caspases 3 or 6) that further induce degradation of cellular components (Fischer *et al.* 2003; for review). Caspase 3 cleaves PARP, a protein involved in DNA repair, and activates DNA fragmentation factor/caspase-activated DNase (DFF/CAD) leading to apoptotic DNA fragmentation. However, apoptotic cell death may occur in a caspase-independent manner (Kroemer and Martin 2005).

In this study, we evaluate the neurotoxicity of *street* heroin in primary cultures of rat cortical neurons. As many drugs of abuse such as amphetamines (Cunha-Oliveira *et al.* 2006; Davidson *et al.* 2001; for review), cocaine (Cunha-Oliveira *et al.* 2006; Nassogne *et al.* 1998; for review) or morphine (Hu *et al.* 2002; Mao *et al.* 2002) have been reported to induce neuronal apoptosis, we characterize the involvement of apoptotic pathways in the neurotoxicity of *street* heroin. Briefly, we show that *street* heroin, in a concentration that slightly decreases metabolic viability, promotes caspase-dependent mitochondrial apoptosis, characterized by a down-regulation of Bcl-2 and a loss of $\Delta\Psi_m$. The decrease in metabolic viability may be attributed to heroin itself, but caspase 3 activation may be due to drug–drug interaction in the cocktail of compounds that were identified in *street* heroin.

Materials and methods

Materials

Optimem medium, neurobasal medium and B27 supplement were supplied by Gibco (Paisley, UK). Chymostatin, leupeptin, antipain, pepstatin A, *N*-acetyl-Ile-Glu-Pro-Asp-*p*-nitroaniline (Ac-IEPD-pNA), *N*-acetyl-Val-Asp-Val-Ala-Asp-*p*-nitroanilide (Ac-VDVAD-pNA), *N*-acetyl-Val-Glu-Ile-Asp-*p*-nitroanilide (Ac-VEID-pNA) and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA) trypsin, soybean trypsin inhibitor (SBTI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), ifenprodil and glutathione ethyl ester (GSH-EE) were supplied by Sigma Chemical Co (St. Louis, MO, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), trolox and *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) were supplied by Calbiochem (Darmstadt, Germany). NT-2 cells were a kind gift from Dr. Russell Swerdlow (University of Virginia Health System, VA, USA). The antibodies anti-caspase 3, anti-caspase 9, anti-Bax and anti-cleaved PARP were supplied by Cell Signaling (Beverly, MA, USA); enhanced chemifluorescence (ECF) and anti-rabbit IgG were obtained from Amersham Biosciences (Piscataway, NJ, USA); MitoTracker Green, Hoechst 33342, Alexa anti-rabbit IgG 488, Alexa anti-mouse IgG 594 and rhodamine 123 (Rh123) were supplied by Molecular Probes (Eugene, OR, USA). Antibodies against the native and denatured forms of cytochrome *c* were obtained from PharMingen (San Diego, CA, USA). (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) was a kind gift from Merck Sharp & Dohme Research Laboratories (Merck & Co. Inc., Whitehouse Station, NJ, USA). Idebenone was a kind gift from Seber (Odivelas, Portugal). Clocinnamox, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzoquinoxaline-7-sulfonamide (NBQX), d-2-amino-5-phosphonopentanoic acid (d-AP-5) and naltrindole were purchased from Tocris (Bristol, UK). Anti-Bcl-2 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). A sample of seizure *street* heroin was provided by the 'Instituto da Droga e da Toxicodependência' (IDT, Lisbon, Portugal). Morphine and codeine hydrochlorides were obtained from Uquipa (Lisbon, Portugal).

Chemical analysis

A street heroin sample was analysed in order to quantify diacetylmorphine, morphine, 6-MAM, codeine and acetylcodeine content. HPLC analyses were conducted on an HPLC system (Merck/Hitachi–LaChrom, Tokyo, Japan) equipped with a diode-array detector (DAD). The analytical column was a commercially pre-packed reverse phase (RP-18) column (250 × 4.0 mm i.d., 5 μm) with pre-column (Waters, Watford, UK). An isocratic elution was performed at a flow rate of 1.5 mL/min and the absorbance was measured at 216 nm. The analysis was carried out, at room temperature (∼20°C), using a solution of 60% 10 mmol/L aqueous ammonium acetate (pH 3) plus 40% acetonitrile as mobile phase. Prior to use, the solutions were filtered and subsequently sonicated for a minimum of 15 min. The volume of sample injected was 20 μL. A calibration curve of six standards was prepared for each of the compounds (heroin, morphine, 6-MAM, codeine and acetylcodeine). Compounds on seizure *street* heroin samples were identified by comparison of their retention times with those of known standards. For this study heroin and analogs were synthesized due to the non-availability of the compounds from commercial sources. Heroin and acetylcodeine were synthesized by the classic method of acetylation, using acetic anhydride and pyridine (Garrido *et al.* 2004a,b) at room temperature (∼20°C). 6-MAM was obtained by deacetylation of the phenolic group of heroin under mild conditions (Garrido *et al.* 2004a). Qualitative and quantitative analysis of the seizure *street* heroin was accomplished using HPLC with UV detection. *Street* heroin maintained the same composition throughout the experiments. Qualitative thin-layer chromatography was used to detect the presence of caffeine and sugars (Moffat 1986; Chiarotti *et al.* 1991; Sharma *et al.* 2005).

Cell culture

Culture of cortical neurons

The frontal cortices of rat embryos (16–17 days) were dissected and the cells were washed with isolation medium (in mmol/L: 120.9 NaCl, 4.83 KCl, 1.22 KH_2PO_4, 25.5 $NaHCO_3$, 13.0 glucose and 10.0 HEPES) containing 0.3% bovine serum albumin (BSA). The cells were sedimented at 180 *g* and dissociated with isolation medium, supplemented with 0.02% trypsin and 0.04 mg/mL DNAse for 10 min at 37°C. Trypsin was inactivated by adding 0.075% SBTI and the cells were centrifuged at 180 *g* for 5 min. The cells were then mechanically dissociated in isolation medium containing 0.012% SBTI and centrifuged at 180 *g* for 5 min. The resulting pellet was resuspended in neurobasal medium with B27 supplement, 0.2 mmol/L glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (in 5% CO_2/95% air) and plated on poly-L-lysine (1 mg/mL) coated multiwells or coverslips. Cortical cultures contained a small percentage of glial cells (less than 10%) as assessed by immunofluorescence using anti-microtubule-associated protein-2 and anti-glial fibrillary acidic protein (not shown). After 6 days in culture, the cells were incubated with *street* heroin (4.3–1280 μg/mL), pure heroin (215 or 840 μmol/L), morphine (4.5 or 17.6 μmol/L) or 6-MAM (47 or 183 μmol/L), for 24 h, unless otherwise specified. *Street* heroin was dissolved in dimethylsulfoxide and the maximal concentration of dimethylsulfoxide (0.2%) used in the experiments was not toxic *per se* (not shown). Where specified, 1 μmol/L z-VAD-fmk, 1 μmol/L naloxone, 1 μmol/L clocinnamox, 1 μmol/L CTOP, 1 μmol/L naltrindole, 2 μmol/L MK-801, 3 μmol/L ifenprodil, 100 μmol/L d-AP-5, 10 μmol/L NBQX, 3 μmol/L idebenone, 100 μmol/L trolox or 100 μmol/L GSH-EE were pre-incubated for 30–60 min and were present in the culture media throughout all the experiments. The concentrations of the compounds tested were chosen based on previous studies (Williams *et al.* 2001; Araujo *et al.* 2003; Gil *et al.* 2003).

Culture of NT-2 cells

Stock cultures of NT2 (human teratocarcinoma) rho^+ cells were purchased from Stratagene (La Jolla, CA, USA). The production of the NT2 rho^0 cell line used in these experiments was previously described (Swerdlow *et al.* 1997). Cells were grown routinely in 75-cm^2 tissue culture flasks in Optimem Medium, supplemented with 10% heat inactivated fetal calf serum, penicillin (50 U/mL) and streptomycin (50 μg/mL). Uridine (50 μg/mL) and pyruvate (200 μg/mL) were also added to rho^0 cell growth medium. The cells were grown and maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. The cells were plated at 0.1×10^6/mL for cell viability assay and incubated with *street* heroin (128 μg/mL) for 24 h.

Cell viability assays

MTT assay

Metabolic cell viability was measured using the MTT reduction assay at 570 nm (Mosmann 1983). The cells were incubated with 0.5 mg/mL MTT in Na^+ medium (in mmol/L: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 5.6 glucose and 20 HEPES, pH 7.4) for 2 h, and the precipitated salt was dissolved with 0.04 mol/L HCl in isopropanol. The capacity of treated cells of reducing the tetrazolium salt was expressed as a percentage of absorbance in control cells.

Lactate dehydrogenase assay

The integrity of the plasma membrane of cortical neurons was determined by monitoring the leakage of lactate dehydrogenase (LDH), by following the rate of conversion of NADH to NAD^+ at 340 nm, according to Bergmeyer and Bernt (1974). Alterations in membrane integrity were expressed as a percentage of LDH release compared with control cells.

Immunocytofluorescence

Cytochrome c

The cells were incubated with MitoTracker Green (1 μmol/L) in Na^+ medium for 1 h. After fixation in 4% *p*-formaldehyde containing 4% sucrose in saline buffer (PBS, in mmol/L: NaCl 137, KCl 2.7, K_2HPO_4 1.4, KH_2PO_4 4.3 at pH 7.4), the cells were incubated with 20 mmol/L glycine. After cell permeabilization in the presence of saponin (0.1% in 20 mmol/L glycine), the cells were incubated with a specific antibody against the native form of cytochrome *c* (PharMingen, 1 : 100 in 0.1% saponin,), which was detected by using a secondary antibody Alexa Fluor 594 anti-mouse IgG (1 : 200 in 0.1% saponin). The cells were visualized in a confocal microscope (Bio-Rad MRC 600, Bio-Rad Laboratories, Hercules, CA, USA).

Cleaved PARP

The cells were fixed in 4% *p*-formaldehyde, permeabilized in 0.2% Triton X-100, for 2 min, and blocked in 3% BSA for 30 min. The fragment resulting from the cleavage of PARP by caspase 3 was detected by using a specific primary antibody (1 : 100 in 3% BSA)

and a secondary Alexa anti-rabbit 488 antibody (1 : 200 in 3% BSA). The nuclei were stained by using Hoechst 33342 (1 μg/mL) and the cells were visualized in an epifluorescence microscope (Zeiss Axioscope).

Fluorimetric evaluation of Rh123 cellular retention

The cells were loaded with 1 μmol/L Rh123 for 10 min, in the dark, at 37°C. The fluorescence (λex 505 nm and λem 525 nm) was recorded for 10 min, before and after permeabilization with 0.5% Triton X-100, in a SPEX Fluorolog spectrometer equipped with a thermostatic water bath. Rh123 retention was determined by the difference between total fluorescence (after permeabilization) and the initial value of fluorescence. Because positively charged Rh123 is retained by the mitochondria under normal conditions, corresponding to a high mitochondrial membrane potential ($\Delta\Psi_m$), a decrease in cellular retention of Rh123 was associated with a decrease in $\Delta\Psi_m$ (Palmeira *et al.* 1996).

Colorimetric evaluation of caspases-like activity

The cells were dissociated in lysis buffer [in mmol/L: 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, 2 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride (PMSF) and 1 μg/mL of protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin A)]. The resulting extracts were frozen and thawed three times, centrifuged at 15 000 *g* for 10 min (4°C), and the protein (supernatant fraction) was assessed by following the Bio-Rad protein assay. The supernatant was tested for the activity of caspases 2, 3, 6, 8 and 9, at 405 nm, after reaction with the respective substrates Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IEPD-pNA and Ac-LEHD-pNA (100 μmol/L, for 2 h at 37°C) as described by Gurtu *et al.* (1997). The results were normalized over the control absorbance value using the same amount of protein (25–40 μg/assay).

Western blotting

Sample preparation

For the analysis of caspases activation by western blotting, the cells were treated as for the caspases activity assay. For the analysis of Bcl-2 and Bax levels, total extracts were obtained using total extraction buffer (in mmol/L: 20 Tris (pH 7), 100 NaCl, 2 EDTA, 2 EGTA, 50 NaF, 1 sodium *o*-vanadate and 0.1 PMSF) supplemented with 100 nmol/L okadaic acid, 1 μg/mL of protease inhibitor cocktail, 0.5% sodium dodecyl sulfate and 0.5% Triton X-100. For the analysis of cytochrome *c* release, cell lysates were homogenized in sucrose buffer (in mmol/L: 250 sucrose, 20 HEPES, 10 KCl, 1.5 $MgCl_2$, 1 EDTA, 1 dithiothreitol and 0.1 PMSF) supplemented with 1 μg/mL of protease inhibitor cocktail. The mitochondrial fraction (P2) was separated by centrifugation at 500 *g* for 12 min (4°C) followed by centrifugation of the resulting supernatant at 12 000 *g* for 20 min (4°C). Cytosolic fraction (P3) was obtained upon protein precipitation of the resulting supernatant with 5% trichloroacetic acid, followed by centrifugation at 15 800 *g* for 10 min and pH neutralization with KOH.

Immunoblotting procedures

Proteins (30–50 μg/sample) were denatured and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12%), and then transferred to a PVDF membrane, which was then incubated with rabbit anti-caspase 3 (1 : 1000), rabbit anti-caspase 9 (1 : 1000), mouse anti-Bcl-2 (1 : 500), rabbit anti-Bax (1 : 1000), mouse anti-cytochrome *c* (1 : 500) and mouse anti-GAPDH (1 : 2500) antibodies. The secondary detection was performed using anti-mouse IgG or anti-rabbit IgG alkaline-phosphatase-bound antibodies (1 : 20 000). The bands were developed with ECF, visualized in a VersaDoc imaging system (Bio-Rad) and quantified using the Quantity One software (Bio-Rad).

Statistical analysis

Data are the mean ± SEM from at least three independent experiments, performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA with Bonferroni *post hoc* test (a $p < 0.05$ was considered significant).

Results

Street heroin dose dependently affects cell viability

Street heroin was shown to be composed by 62% heroin, 12% 6-MAM, 1% morphine, with trace quantities of codeine, acetylcodeine (less than 0.2% each), starch and caffeine, and other unidentified diluents and adulterants (about 25%). The results are in accordance with the data published by the U.S. Drug Enforcement Administration (DEA), which show that the purity of the majority of *street* heroin samples ranges from 10% to 70%.

In order to define experimental conditions of moderate neurotoxicity, we performed a dose-response analysis of cell viability, following the MTT reduction assay (Fig. 1a) and the LDH release assay (Fig. 1b), after incubation with *street* heroin, for 24 h. *Street* heroin induced a dose-dependent decrease in metabolic viability, showing an IC_{10} value of 128 μg/mL, corresponding to 215 μmol/L heroin, 47 μmol/L 6-MAM and 4.5 μmol/L morphine. Under these conditions, plasma membrane integrity, determined by the LDH leakage assay, was maintained (Fig. 1b). The IC_{50} was calculated to be 500 μg/mL, corresponding to 840 μmol/L heroin, 183 μmol/L 6-MAM and 17.6 μmol/L morphine. Membrane integrity was only affected by high concentrations of *street* heroin (Fig. 1b), suggesting that, for concentrations up to 427 μg/mL, the neurotoxicity induced by *street* heroin is independent of necrosis. As the *street* heroin sample was not pure, we tested the effect of the identified components (pure heroin, 6-MAM and morphine) on metabolic viability (Fig. 1c). While no toxic effects were induced by either 6-MAM or morphine, pure heroin induced a similar decrease in MTT reduction, compared to treatment with *street* heroin. Despite acetylcodeine and codeine were present in very low quantities in *street* heroin, we tested the effect of these compounds on neuronal viability, as acetylcodeine has been reported to be more toxic than heroin (Soine 1986). Acetylcodeine (1–

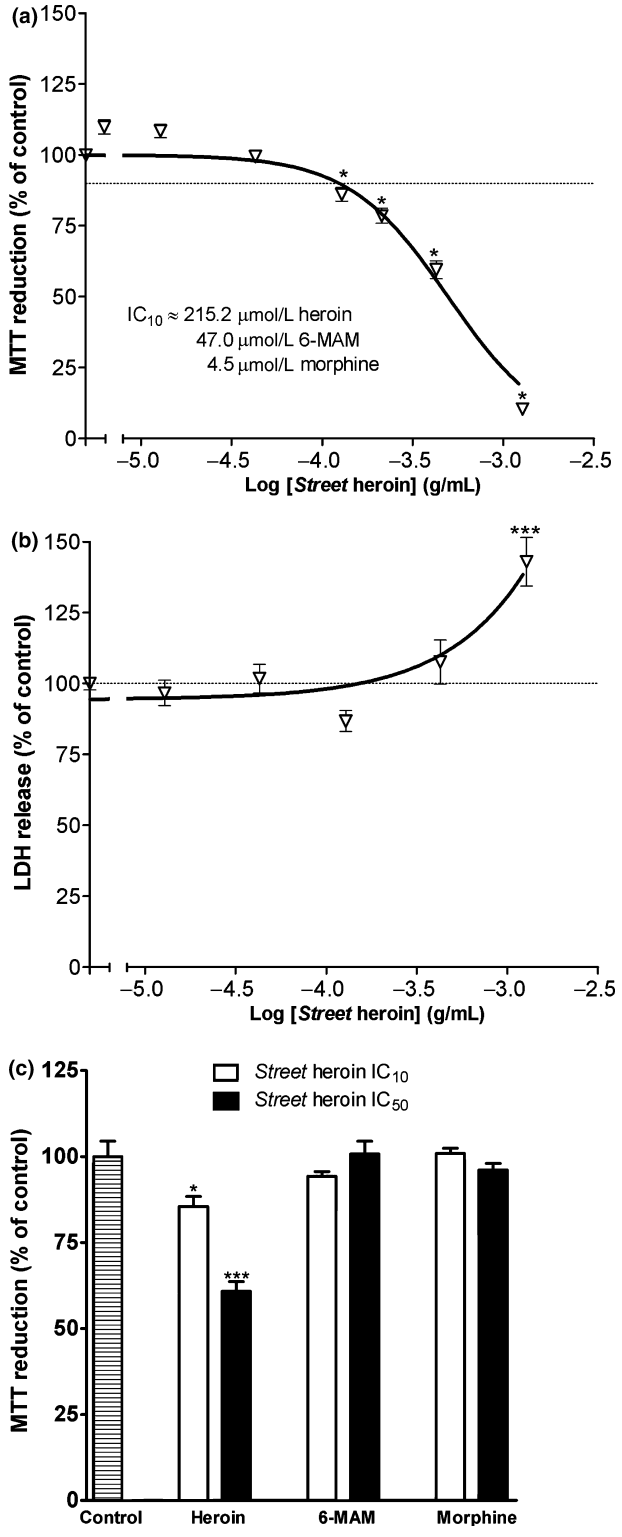

Fig. 1 Analysis of cell viability upon exposure to *street* heroin. Cortical neurons were incubated with increasing concentrations of *street* heroin (4.3–1280 μg/mL), for 24 h, and the dose-dependent changes in cell viability were evaluated by (a) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay, which evaluates changes in metabolic viability, and by (b) lactate dehydrogenase (LDH) leakage assay, in order to analyse changes in membrane integrity. Data are the mean ± SEM of three to five experiments performed in duplicate or triplicate. The dotted line in (a) represents the IC_{10} interpolation, corresponding to 128 μg/mL (215.2 μmol/L heroin, 47.0 μmol/L 6-monoacetylmorphine and 4.5 μmol/L morphine). Statistical significance: $^{*}p < 0.001$ as compared with the control. (c) Analysis of cell viability upon exposure to pure heroin (215 and 840 μmol/L), 6-monoacetyl morphine (6-MAM) (47 and 183 μmol/L) and morphine (4.5 and 17.6 μmol/L) using the concentrations found in *street* heroin, corresponding to IC_{10} and IC_{50}. Statistical significance: $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with the control.

100 μmol/L) did not significantly affect cortical neuron metabolic viability, whereas codeine (10–30 μmol/L) induced a slight decrease (by about 10%) in MTT reduction (not shown).

Caspases of the mitochondrial apoptotic pathway are preferentially activated by *street* heroin

We investigated the involvement of apoptotic signaling, namely caspases activation, induced by the concentration of *street* heroin corresponding to the IC_{10} values determined in Fig. 1a. Initiator caspases of the mitochondrial pathway, caspases 2 and 9, were significantly activated after 12 h of *street* heroin exposure (Fig. 2a), whereas caspase 8 was only slightly activated at a later time point (24 h), indicating that the extrinsic pathway was not involved in the initiation of the apoptotic cascade. Caspase 3 activity in cells treated with *street* heroin was significantly increased after 12 h exposure, when compared with the control (Fig. 2a), and remained elevated up to 24 h. The activity of the effector caspase 6 only slightly increased in 24 h *street* heroin-treated cells (not shown). Proteolytic processing of caspases 9 and 3 was confirmed by western blotting (Figs 2b and c). Forty-eight hours exposure to *street* heroin resulted in a massive cell death, as indicated by a large decrease in MTT reduction and a major decrease in caspase 3 activity (data not shown). *Street* heroin-induced increase in caspase 3 activity at 24 h was completely prevented in the presence of 1 μmol/L z-VAD-fmk, a non-selective caspase inhibitor (Fig. 2d). Pure heroin (215 μmol/L) only slightly activated caspase 3 (1.4-fold above the control, Fig. 2e), suggesting that caspase 3 may be further activated by other substances present in *street* heroin and/or that combination of *street* heroin components act synergistically to cause apoptotic cell death.

DNA fragmentation induced by *street* heroin is completely dependent on caspase activity

According to data on Fig. 2, caspase-dependent apoptotic cell death seems to be involved in the neurotoxic effects of *street* heroin. This was further evaluated by analyzing the occurrence of nuclear apoptotic morphology and PARP cleavage in cells treated with the drug of abuse (Fig. 3).

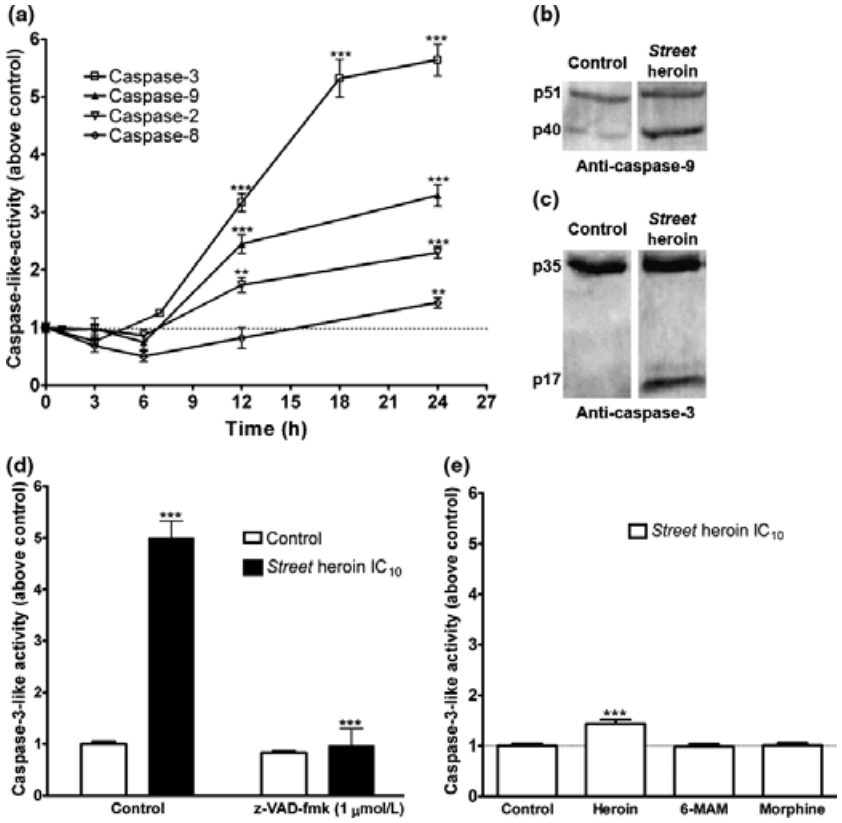

Fig. 2 Caspases activation in cells incubated with *street* heroin. (a) Time course analysis of caspase activation. Cellular extracts were tested for the activity of caspases 3, 9, 2 and 8, by determining the extent of cleavage of caspases substrates: *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA), *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA), *N*-acetyl-Val-Asp-Val-Ala-Asp-*p*-nitroanilide (Ac-VDVAD-pNA) and *N*-acetyl-Ile-Glu-Pro-Asp-*p*-nitroanilide (Ac-IEPD-pNA), respectively. The cells were incubated with *street* heroin (IC_{10}), for 1–24 h. Pro-caspases 3 (b) and 9 (c) processing, after 24 h of exposure to *street* heroin, was also examined by western blotting. (d) Complete inhibition of *street* heroin-induced caspase 3 activation in the presence of 1 μmol/L z-VAD-fmk. (e) Caspase 3 activity upon 24 h exposure to 215 μmol/L pure heroin, 47 μmol/L 6-monoacetyl morphine (6-MAM) or 4.5 μmol/L morphine, corresponding to *street* heroin IC_{10}. Data are the mean ± SEM of the fold increase above control absorbance values of three experiments performed in duplicate. Statistical significance: $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control.

Street heroin induced an increase (by about 17%) in the number of cells showing fragmented or condensed DNA, as evaluated by nuclear staining with Hoechst 33342 (Fig. 3). The number of cells showing immunoreactivity to PARP, cleaved on the caspase cleavage site, also increased (Fig. 3a), reflecting the activation of caspase 3. DNA fragmentation induced by *street* heroin was completely prevented by 1 μmol/L z-VAD-fmk (Fig. 3b), a concentration previously shown to prevent the increase in caspase 3 activity induced by *street* heroin (Fig. 2d). These data support an important role for caspases in the neurotoxic effects of *street* heroin.

Caspase activation is not mediated by opioid or ionotropic glutamate receptors and appears to be independent of oxidative stress

We further investigated the mechanisms leading to caspase 3 activation induced by *street* heroin by analyzing the involvement of opioid and ionotropic glutamate receptors and the contribution of reactive oxygen species (ROS). The involvement of opioid receptors was determined by using a non-selective opioid receptor antagonist, naloxone, the mu-opioid receptor antagonists, clocinnamox and CTOP, and the delta-opioid receptor antagonist, naltrindole (Table 1a). Under these conditions, a partial prevention of caspase 3 activation was observed in the presence of naloxone (about 30%). Naloxone also prevented (by about 25%) the appearance of nuclear apoptotic morphology induced by *street* heroin (not shown). However, the selective opioid antagonists did not significantly affect caspase 3 activity induced by *street* heroin. These data suggest that opioid receptors do not mediate *street* heroin neurotoxicity, and that naloxone may prevent caspase 3 activation, and apoptosis, by an opioid receptor-independent mechanism, as previously reported (Liu *et al.* 2002).

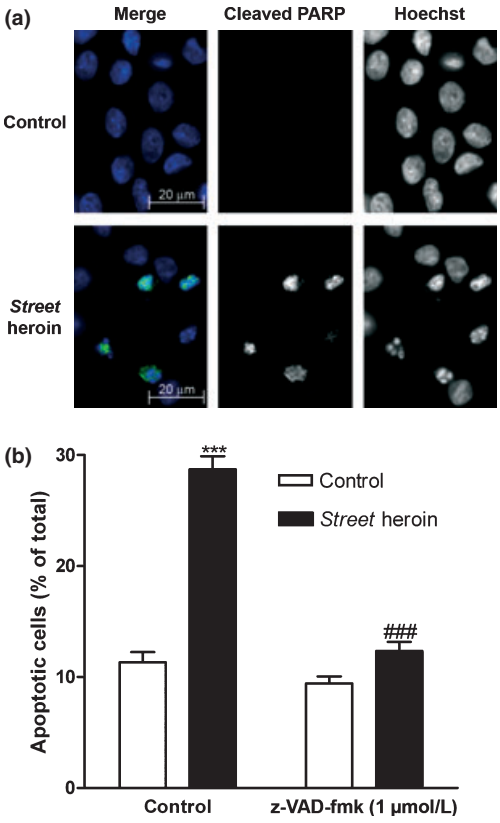

Fig. 3 Analysis of nuclear apoptotic morphology in cortical neurons exposed to *street* heroin. (a) Immunocytochemical analysis of apoptotic morphology. Apoptotic cell death upon exposure to *street* heroin (IC_{10}, for 24 h) was evaluated by labeling cleaved PARP, using a specific antibody (in green), and by nuclear morphology following Hoechst 33342 staining (in blue). (b) Complete prevention of DNA fragmentation induced by *street* heroin, in the presence of 1 μmol/L z-VAD-fmk. About 500–700 cells were counted per coverslip. Data are the mean ± SEM of three to four experiments performed in duplicates. Statistical significance: ***$p < 0.001$ compared with the control, ###$p < 0.001$ compared with *street* heroin alone.

Moreover, as apoptotic cell death frequently depends on excitotoxic events mediated by ionotropic glutamate receptors, and these receptors were reported to be present in embryonic cortical neurons (Babb *et al.* 2005), we also tested the effect of the NMDA receptor antagonists MK-801, ifenprodil and d-AP-5, and the AMPA receptor antagonist NBQX, in the activation of caspase 3 induced by *street* heroin (Table 1b). The results indicate that ionotropic glutamate receptors are not involved in *street* heroin neurotoxicity, as the antagonists of NMDA or AMPA receptors did not prevent *street* heroin-induced caspase 3 activation.

Because apoptotic cell death has been associated with oxidative stress, we tested whether the antioxidants idebenone (a benzoquinone derivative of coenzyme Q10), trolox (an analogue of vitamin E) or GSH-EE (a cell-permeable form of reduced glutathione) protected against *street* heroin-induced apoptosis (Table 1c). None of the antioxidants tested prevented *street* heroin-induced caspase 3 activation, indicating that oxidative stress is not involved in the apoptotic process induced by this drug of abuse.

Street heroin induces cytochrome *c* release and mitochondrial dysfunction

Initiator caspases (2 and 9) activated by *street* heroin at early time points (Fig. 2a) have been largely associated with the mitochondrial apoptotic pathway. Thus we analysed whether the release of cytochrome *c* and mitochondrial dysfunction contributed to the neurotoxic effects of *street* heroin.

Analysis of cytochrome *c* release by immunocytochemistry (Fig. 4a) showed a decrease in co-localization between cytochrome *c* (labeled with a specific antibody) and the mitochondria (labeled with MitoTracker Green) upon treatment with *street* heroin, indicating a decrease in mitochondrial cytochrome *c* content. *Street* heroin-mediated release of cytochrome *c* to the cytosol was further evidenced by western blotting, showing a decrease in mitochondrial cytochrome *c* content (by about 60%) and a consequent increase in the cytosolic fraction (Fig. 4b). An increase in mitochondrial permeability, responsible for cytochrome *c* release, has been associated with a decrease in $\Delta\Psi_m$. Thus, we evaluated the changes in $\Delta\Psi_m$, induced by *street* heroin (Fig. 4c), by following the cellular retention of Rh123, described to be higher in cells maintaining $\Delta\Psi_m$ (Palmeira *et al.* 1996). Our data suggest that exposure to *street* heroin causes a great decrease in $\Delta\Psi_m$ (by about 55%).

Because *street* heroin neurotoxicity involved mitochondrial dysfunction, we investigated the requirement of a functional respiratory chain. Thus, we analysed *street* heroin neurotoxicity in NT-2 rho^0 cells, which do not possess a functional electron transport chain (Cardoso *et al.* 2001), in comparison with NT-2 rho^+ cells (Fig. 4d). The toxicity of *street* heroin in NT-2 rho^0 cells was not significantly different compared with NT-2 rho^+ cells, suggesting that *street* heroin neurotoxicity is independent of an interaction with a functional mitochondrial respiratory chain.

Pro-apoptotic proteins such as Bax have been shown to mediate the release of cytochrome *c*, whereas anti-apoptotic proteins such as Bcl-2 modulate the activity of pro-apoptotic proteins. Thus, we determined the total levels of Bcl-2 and Bax, by western blotting, after exposure to *street* heroin (Figs 4e and f). We found a significant decrease (by about 37%) in Bcl-2 levels (Fig. 4e), without major changes in the levels of Bax (Fig. 4f) in cortical neurons treated with *street* heroin. These results indicate that *street* heroin induces a decrease in Bcl-2/Bax ratio, favoring the proapoptotic activity of Bax, which could help to explain the mechanism involved in cytochrome *c* release induced by *street* heroin. The fact that *street* heroin neurotoxicity seems to be independent of a functional mitochondrial respiratory chain,

Table 1 Analysis of cellular mechanisms involved in caspase 3 activation in cells incubated with *street* heroin (IC_{10})

		caspase 3-like activity (above control)	caspase 3-like activity (above heroin)
	Antagonists	− *Street* heroin	+ *Street* heroin
(a) Opioid receptors	None	1.00 ± 0.03	1.00 ± 0.02
Non-selective	Naloxone (1 μmol/L)	0.87 ± 0.12	0.71 ± 0.03**
Micra	Cloccinamox (1 μmol/L)	0.98 ± 0.05	0.93 ± 0.06
	CTOP (1 μmol/L)	0.92 ± 0.16	0.94 ± 0.05
Delta	Naltrindole (1 μmol/L)	1.04 ± 0.06	0.97 ± 0.07
(b) Glutamate receptors	None	1.00 ± 0.02	1.00 ± 0.02
NMDA	MK-801 (2 μmol/L)	1.27 ± 0.10	1.01 ± 0.06
	Ifenprodil (3 μmol/L)	1.24 ± 0.12	1.11 ± 0.11
	AP-5 (100 μmol/L)	1.11 ± 0.19	1.21 ± 0.08
AMPA	NBQX (10 μmol/L)	1.22 ± 0.12	0.97 ± 0.12
(c) Antioxidants	None	1.00 ± 0.08	1.00 ± 0.04
	Idebenone (3 μmol/L)	1.48 ± 0.16	1.08 ± 0.09
	Trolox (100 μmol/L)	1.02 ± 0.17	1.00 ± 0.03
	GSH (100 μmol/L)	1.30 ± 0.12	1.01 ± 0.09

(a) The involvement of opioid receptors was investigated by testing the effect of a non-selective antagonist (1 μmol/L naloxone) and antagonists of mu (1 μmol/L clocinnamox and 1 μmol/L CTOP) and delta (1 μmol/L naltrindole) opioid receptors. (b) The involvement of ionotropic glutamate receptors in caspase 3 activation was investigated using antagonists of NMDA (2 μmol/L MK-801, 3 μmol/L ifenprodil or 100 μmol/L d-AP-5) and AMPA (10 μmol/L NBQX) receptors. (c) The involvement of reactive oxygen species in caspase 3 activation induced by *street* heroin was investigated using antioxidants idebenone (3 μmol/L), trolox (100 μmol/L) and glutathione ethyl ester (100 μmol/L GSH-EE). Data are the mean ± SEM of three experiments performed in duplicate. Statistical significance: $^{**}p < 0.01$, compared with *street* heroin alone.

suggests that *street* heroin-induced decrease in $\Delta\Psi_m$ in cortical neurons may be due to the formation of mitochondrial permeability transition or Bax-composed pores. As it was previously described that Bax channel inhibitors can block the decrease in $\Delta\Psi_m$ induced by a pro-apoptotic inducer (Hetz *et al.* 2005), a decrease in Bcl-2/Bax may lead to mitochondrial permeabilization and consequent loss of $\Delta\Psi_m$.

Discussion

In this study, we show that *street* heroin induces cell death by a mitochondrial-dependent apoptotic pathway, initiated by caspases 2 and 9, and involving cytochrome *c* release, loss of mitochondrial potential and down-regulation of Bcl-2. This process is not dependent on the activation of opioid or ionotropic glutamate receptors, nor is it dependent on oxidative stress. Due to the fact that the *street* heroin sample was a mixture, the effects observed in this study may be either due to heroin or to the combination of *street* heroin components, which can act synergistically.

Acetylcodeine has been reported to be more toxic than heroin (Soine 1986). However, in our experimental conditions, acetylcodeine did not significantly affect cortical neurons metabolic viability, whereas codeine induced a slight decrease in MTT reduction, when used in higher concentrations than those achieved in *street* heroin. Noscapine (20 μmol/L), another alkaloid present in opium extracts, has been shown to induce apoptosis in HeLa cells and thymocytes (Ye *et al.* 1998). In addition to opium substances, *street heroin* often contains some adulterants, namely paracetamol, acetaminophen, caffeine and theophylline (Zhang *et al.* 2004), which may also exhibit toxic effects. Very toxic adulterants are not usually detected in this type of samples because when present, they are found in very low concentrations (Chiarotti *et al.* 1991; Sharma *et al.* 2005).

The mechanisms of cell death induced by heroin are largely unknown. Fecho and Lysle (2000) showed that heroin decreased the number of leukocytes in the rat spleen, which presented several apoptotic features such as annexin V labeling and DNA fragmentation. In contrast, the cytotoxicity of morphine, a metabolite of heroin that may coexist in heroin solutions (Hutchinson and Somogyi 2002), has been investigated by several groups. Hu *et al.* (2002) showed that morphine increased the number of apoptotic microglia and neurons. This process of cell death was blocked by naloxone and involved caspase 3 activation and DNA fragmentation. Other authors (Mao *et al.* 2002) showed that prolonged morphine administration increased rat spinal neuronal apoptosis, with up-regulation of caspase 3 and Bax and down-regulation of Bcl-2 in the spinal dorsal horn. The non-selective caspase inhibitor z-VAD-fmk and the caspase 3 inhibitor Ac-DEVD-CHO blocked neuronal apoptosis in-

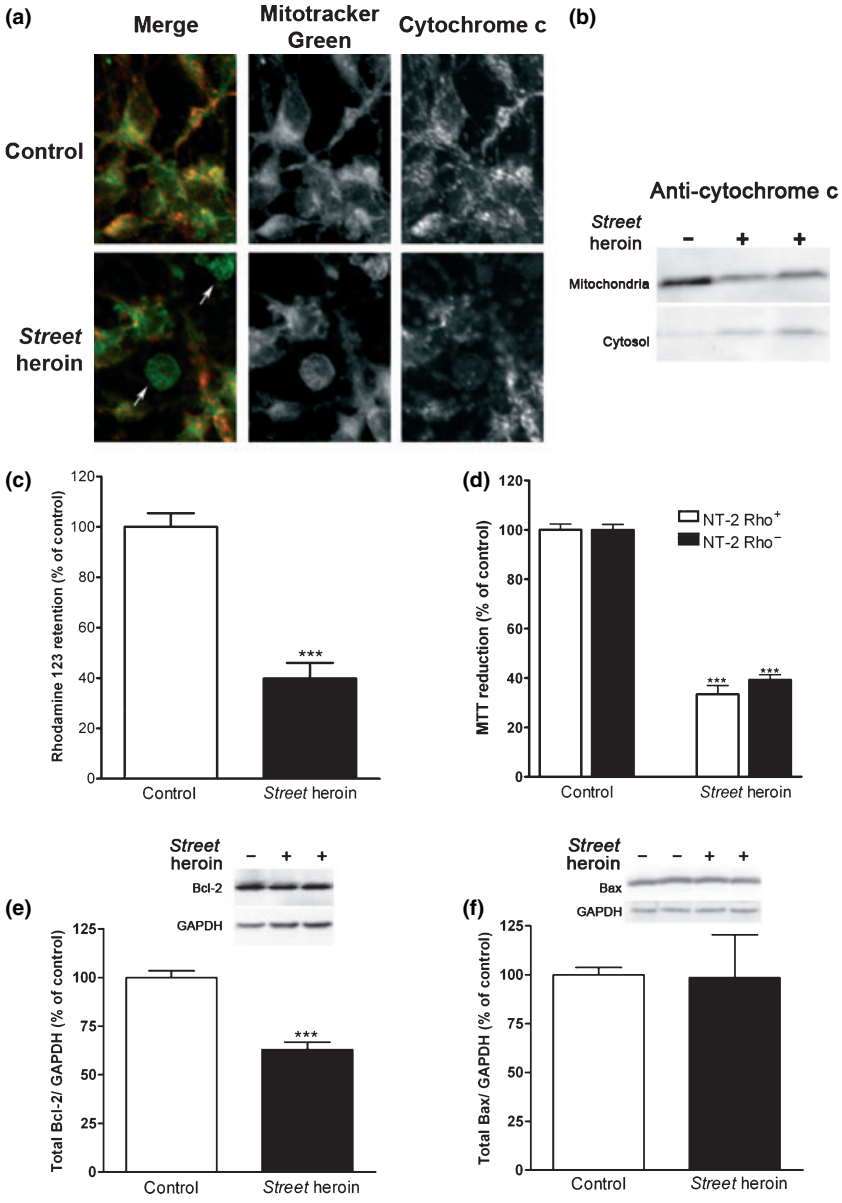

Fig. 4 Analysis of cytochrome *c* release and mitochondrial dysfunction after exposure to *street* heroin (IC_{10}, for 24 h). (a and b) Analysis of cytochrome *c* release from the mitochondria, by immunocytofluorescence (a), using MitoTracker Green to label the mitochondria (in green) and an antibody against the native form of cytochrome *c* (in red) or by western blotting (b) using subcellular fractions corresponding to the same samples. (c) Analysis of rhodamine 123 (Rh123) retention capacity of cortical neurons after exposure to the drug of abuse. Rh123 retention capacity was used to evaluate $\Delta\Psi_m$ in cortical neurons incubated with *street* heroin (IC_{10}). The cells were incubated with 1 μmol/L Rh123, for 10 min. The fluorescence of the probe was recorded before (F_i) and after (F_f) cell permeabilization with Triton X-100. Rh123 retention capacity was calculated by the difference: F_f–F_i. (d) Analysis of *street* heroin cytotoxicity in NT-2 rho$^+$ and rho^0 cells by following the cellular capacity to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) after exposure to *street* heroin (IC_{10}, 24 h). (e and f) Analysis of total Bcl-2 and Bax levels by western blotting. Images are representative of at least three experiments performed in duplicate. Quantitative data are the mean ± SEM of at least three experiments performed in duplicate or triplicate. Statistical significance: ***$p < 0.001$ compared with the control.

duced by morphine. Moreover, in this study, the apoptotic pathway was reported to be mediated by the NMDA receptors (Mao *et al.* 2002). In another study, Yin *et al.* (1999) showed that morphine induced the expression of Fas and promoted Fas-L-mediated apoptosis of lymphocytes. This effect was blocked by naloxone, suggesting the involvement of the opioid receptors. Furthermore, Singhal *et al.* (2002) showed that morphine-induced apoptosis of T-cells, with activation of caspases 3, 8 and 10 and PARP cleavage. This apoptotic pathway was reported to be mediated by c-*jun* N-terminal kinase activation. Together, these studies suggest that morphine is able to induce apoptosis in several cell types, including neurons, in a process mediated by opioid receptors. Another study (Jiang *et al.* 2003) showed that morphine, heroin and cocaine up-regulate mouse double minute-2 (MDM2) in several brain regions. MDM2 is known for suppressing the activity of p53. Increased MDM2 may reflect an increase in p53 activity induced by the drugs of abuse (Jiang *et al.* 2003). The neurotoxicity and apoptotic cell death induced by morphine were also suggested to be mediated by an increase in c-*jun* N-terminal kinase 3 expression (Fan *et al.* 2003). According to our results only pure heroin slightly contributed for caspase 3 activation and loss of cell viability, whereas morphine did not exhibit toxic effects.

Cortical neurons were previously reported to express both mu and delta opioid receptors (Lee *et al.* 2002). In the present work, although we observed a reduction of caspase 3 activation (by about 30%) and a reduction in apoptotic nuclear morphology (by about 25%, data not shown) in the

presence of naloxone, the involvement of opioid receptors in apoptotic cell death induced by *street* heroin was not corroborated by using specific-opioid receptor antagonists (Table 1a). Naloxone was previously shown to mediate neuroprotection independently of the interaction with opioid receptors (Liu *et al.* 2002). Moreover, the stereoisomer (+)-naloxone, which has no activity as an opioid antagonist, effectively inhibits microglial activation and has been demonstrated to be neuroprotective (Liu *et al.* 2002). It was previously suggested that naloxone could also directly interact with the nicotinic receptor (Tome *et al.* 2001). In our cellular model, *street* heroin's neurotoxicity is largely independent of opioid receptors, and naloxone may prevent apoptotic cell death induced by *street* heroin by a mechanism independent of the interaction with opioid receptors. Morphine was previously reported to induce loss of $\Delta\Psi_m$ in human glioma cells by a mechanism mediated by naloxone-sensitive receptors (Mastronicola *et al.* 2004). Thus, naloxone-mediated neuroprotection observed in the present work may be related with the inhibition of *street* heroin-induced loss of $\Delta\Psi_m$. In addition to these results, the Adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase activator forskolin (1–30 μmol/L) was not able to prevent *street* heroin-induced caspase 3 activation (data not shown), suggesting that the mechanism involved in apoptosis induced by *street* heroin is not dependent on PKA inhibition, which is known to occur upon activation of opioid receptors. Moreover, the fact that opioid receptors are not involved in *street* heroin-induced apoptosis is in agreement with the lack of major apoptotic effects of the opioid components identified in our *street* heroin sample (Fig. 2e).

Ionotropic glutamate receptor activation, frequently associated with excitotoxic cell death, was not involved in *street* heroin-induced apoptosis either, because antagonists of the NMDA or AMPA receptors were not able to rescue caspase 3 activation induced by heroin (Table 1b). Moreover, nicotine, an agonist of the nicotinic acetylcholine receptor, previously shown to be neuroprotective in cortical cell cultures through a decrease in caspase activation and inhibition of apoptosis induced by oxygen deprivation (Hejmadi *et al.* 2003), did not prevent *street* heroin-induced caspase 3 activation, when used at 10–30 μmol/L (data not shown).

Other mediators of cell death involving mitochondrial dysfunction are ROS. Previously, other authors reported that *in vivo* exposure of mice to heroin induced oxidative stress (Pan *et al.* 2005; Qiusheng *et al.* 2005). However, in the present study, the antioxidants idebenone, trolox and GSH-EE did not prevent *street* heroin-induced caspase 3 activation (Table 1c), indicating that, in our conditions, an increase in ROS levels is not upstream of caspase 3 activation. In addition, although nitric oxide synthase activation has been reported to mediate mitochondrial dysfunction and apoptosis in cortical neurons subjected to glutamate (Almeida and Bolanos 2001), in this work the nitric oxide synthase inhibitors *N*-omega-nitro-L-arginine methyl ester (100–500 μmol/L) or 7-nitroindazole (10–100 μmol/L) were also unable to prevent *street* heroin-induced caspase 3 activation (data not shown).

The time course of apoptotic events in this work suggests the activation of caspases 2 and 9 upon 6–12 h of *street* heroin exposure, resulting in the activation of caspase 3. Caspase 3 activity is also suggested by the occurrence of PARP cleavage and DNA fragmentation. In addition, upon 24 h exposure to *street* heroin, metabolic viability was decreased by about 10% but there were no evidences of changes in plasma membrane integrity, suggesting that necrosis was not involved at this time point. Although the mechanism underlying *street* heroin-induced apoptosis does not seem to involve the activation of plasma membrane receptors (opioid or glutamate receptors), it is likely to be explained by intracellular drug–drug interactions of one or more components of *street* heroin. This interaction is likely to be directed to Bcl-2 expression and to the mitochondria, resulting in the down-regulation of Bcl-2 and the loss of $\Delta\Psi_m$. These changes may help to explain cytochrome *c* release. Moreover, *street* heroin-induced loss of $\Delta\Psi_m$ in cortical neurons was similar to that induced by amphetamine in cortical neurons (Cunha-Oliveira *et al.* 2006), although activation of all of the caspases studied was more evident upon treatment with *street* heroin. Furthermore, a possible interaction of heroin (or other components of *street* heroin) with the mitochondria does not require a functional mitochondrial respiratory chain, as the drug was as toxic in NT-2 rho^0 cells as in NT-2 rho^+ cells.

Concluding, we demonstrate the importance of the mitochondrial apoptotic pathway involving caspases activation in the neurotoxicity of *street* heroin, which may be caused either by the drug itself or by the cocktail of *street* heroin components that can act synergistically.

Although further studies are required to determine the mitochondrial changes upon *in vivo* heroin administration, the intrinsic apoptotic pathway appears to be an important target for neuroprotective strategies in heroin addicted individuals, for whom the use of impure heroin represents an increased neurotoxic risk.

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