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Inhibition of apoptotic signaling in spermine-treated vascular smooth muscle cells by a novel glutathione precursor

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Abstract

Chronic kidney disease (CKD) is a public health problem, mediated by hemodynamic and non-hemodynamic events including oxidative stress. We investigated the effect of two glutathione (GSH) precursors, N-acetyl-cysteine (NAC) and cystine as the physiologic carrier of cysteine in GSH with added selenomethionine (F1) in preventing spermine (uremic toxin) induced apoptosis in cultured human aortic vascular smooth muscle cells (VSMC). VSMCs exposed to spermine (15 μ M) with or without antioxidants (dose 50, 100, 200 and 500 μ g/ml) were assessed for apoptosis, c-Jun-NH₂-terminal kinase (JNK) activation and inducible nitric oxide synthase (iNOS) induction, and activation of intrinsic pathway signaling. Spermine exposure resulted in activation of JNK and iNOS induction, and apoptosis. NAC and F1 (dose range 50–500 μ g/ml) attenuated spermine-induced acceleration of VSMC apoptosis, but only F1 (at 200 and 500 μ g/ml) maintained spermine-induced apoptosis at control levels. Spermine-induced JNK activation was prevented by 200 μ g/ml of both NAC and F1, while iNOS induction was blocked only by F1. Notably, the adverse effects of spermine on BAX/BCL-2 ratio, cytochrome c release, and caspase activation was fully attenuated by F1. In conclusion, F1 was more effective than NAC in preventing spermine-induced apoptosis and downstream changes in related signal transduction pathways in VSMCs. Further studies are needed to examine the effect of these compounds in preventing CKD-associated vascular disease.

Keywords

Spermine; Apoptosis; vascular smooth muscle cells; antioxidants; chronic kidney disease

1. Introduction

Chronic kidney disease (CKD) is an emerging global public health problem, which is associated with rising costs and extremely high morbidity and mortality. CKD is now recognized as one of the most consistent and robust predictors of premature cardiovascular disease (CVD). The link between CKD and CVD appears to be multifactorial. Potential mechanism through which CKD promotes CVD include, but are not limited to, hemodynamic (e.g. increased blood pressure), and non-hemodynamic events including oxidative stress, inflammation, dyslipidemia, and accelerated apoptosis (Clarke and Bennett,

2006; Clarke et al, 2006; Himmelfarb et al, 2002; Jones 2006, Mann et al, 2007; Vaziri et al, 2000; 2007).

The associated oxidative stress promotes lipid/lipoprotein oxidation, monocyte activation, adhesion and migration, and uptake of oxidized lipids and lipoproteins by macrophages and resident cells in the artery wall leading to atherosclerosis (Himmelfarb et al, 2002). In addition oxidative stress contributes to macrophage and vascular smooth muscle cells (VSMC) apoptosis, plaque instability, rupture and thrombosis (Abrass, 2004; Clarke and Bennett, 2006; Clarke et al, 2006). Spermine, a polyamine product, is a reputed uremia toxin which has been implicated as a potential mediator of a number of the CKD-associated clinical abnormalities (Radtke et al, 1981; Takano et al, 2005). Spermine administration has been shown to increase intracellular reactive oxygen species (ROS) in primary human cells and malignant tumor cell lines, and induces apoptosis by activating the mitochondria-dependent intrinsic pathway signaling (Schiller et al, 2005).

Normally cells are equipped with an elaborate antioxidant defense system consisting of numerous antioxidant enzymes and scavenger molecules which work in concert to protect against injury and cell death via detoxification of ROS and xenobiotics. The most abundant endogenous antioxidant in eukaryotic cells is the tripeptide glutathione which is critical for the maintenance of the cellular redox state (Han et al, 2006; Wu et al, 2004). In fact, selective inhibition of enzymes of the glutathione redox cycle heightens the susceptibility to ROS-mediated cell injury (Harlan et al, 1984; Vazari et al, 2004). Reduced GSH exerts potent antioxidant actions by directly scavenging ROS and by serving as the substrate in reactions catalyzed by major antioxidant enzymes such as glutathione peroxidase (Han et al, 2006; Wu et al, 2004). GSH protects cells against oxidative stress by maintaining oxidative balance in the cytosol (85% to 90%), with a smaller portion functioning in organelles such as mitochondria, nuclear matrix, and peroxisomes (Han et al, 2006; Harlan et al, 1984; Vazari et al, 2000; Wu et al, 2004). CKD results in depletion of reduced glutathione and other thiols and marked reduction of reduced glutathione (GSH)/oxidized glutathione (GS-SG) ratio (Han et al, 2006). Depletion of GSH has been implicated in the cellular apoptotic response (Kohen and Nysks, 2002; Kyaw et al, 2004). N-acetyl-cysteine (NAC), a glutathione precursor has shown promise as an effective antioxidant (Drager et al, 2004; Lopez et al, 1998; Shimada et al, 2009) although clinical findings have been inconsistent (Kshirsagar et al, 2004; Miner et al, 2004; Nallamotheu et al, 2004).

We hypothesized that strategies aimed at GSH repletion may attenuate CKD related accelerated apoptosis. To this end we assessed the efficacy of NAC and a novel GSH precursor on preventing uremic toxin induced apoptosis and related signal transduction pathways in spermine treated VSMC as a model of CKD related vascular disease.

2. Materials and Methods

2.1. Antioxidants

N-acetylcysteine (Sigma-Aldrich) and FT061452/RE39734 TM or F1, a GSH precursor with cystine replacing cysteine and selenomethionine added (1.4 micrograms of selenomethionine per 500 mg of cystine; ProImmune®, Rhinebeck, NY).

2.2. Cell culture

Human Aortic Smooth Muscle Cells (Lonza Walkersville, Walkersville, MD) were cultured according to manufacturers' instructions and using their special Bullet Kit medium (Lonza). The cells were grown to desired confluency and subjected to one of the following treatments for up to 72 h: 1) spermine (Sigma-Aldrich Corp, St Louis, MO); 2) spermine + NAC (Sigma-Aldrich); 3) spermine + F1; 4) control. The 15 μ M concentration of spermine used

in the present study was based on the results of our preliminary analyses, which showed 25–35% VSMC apoptosis after 24 h of exposure. NAC and F1 were tested in the 50–500 µg/ml dose range, and we found 200 µg/ml of NAC and F1 were optimal for studying signal transduction pathways

2.3. c-Jun-NH₂-terminal kinase (JNK) activation

Activation of JNK was measured by TiterZyme® Enzyme Immunometric Assay kit (Assay Designs INC., Ann Arbor, MI), as described previously (Jia et al, 2009). The kit uses a monoclonal antibody to JNK immobilized on a microtiter plate to bind the JNK in the standards or samples. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to phospho-JNK is added. This antibody binds to the phospho-JNK captured on the plate. After a short incubation, the excess antibody is washed out and streptavidin conjugated to HRP is added, which binds to the biotinylated monoclonal phospho-JNK antibody; this generates signal for chromogenic substrate. The color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of phospho-JNK in the samples.

2.4. Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL)

In situ detection of cells with DNA strand breaks was performed in paraformaldehyde-fixed cells by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique (Braga et al, 2008; Sinha-Hikim et al, 2007), using a ApopTag-peroxidase kit (Chemicon International, Inc., San Francisco, CA). Enumeration of TUNEL-positive nuclei was carried out in slides stained with ApopTag-peroxidase kit using an American Optical Microscope with an X40 objective and a pair of X10 eyepieces. Methyl-green was used as a counterstain to detect non apoptotic nuclei. The rate of apoptosis was expressed as the percentage of the TUNEL-positive nuclei per total nuclei (apoptotic plus non apoptotic) counted per microscopic field.

2.5. Immunocytochemistry

The cells were grown in Poly-D-Lysine, Collagen 1, and fibronectin-coated chambered slides (Fisher Scientific, Pittsburgh, PA), and each well was treated either as control, with spermine, or spermine and one of the antioxidants as mentioned above. Confluent cultures (75%) of VSMC were fixed with 2% paraformaldehyde and immunostained as described previously (Braga et al, 2008; Brown et al, 2009; Sinha-Hikim et al, 2007). Primary antibodies included rabbit polyclonal caspase 9 (1:100), iNOS (1:50), Cytochrome c (1:50), and BCL-2 (1:100) antibodies from Santa Cruz Biotechnology, Santa Cruz, CA. Immunoreactivity was detected using biotinylated anti-rabbit or anti-mouse IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex, and visualized with diaminobenzidine tetrahydrochloride (DAB) as per the manufacturer's instructions (VECTASTAIN Elite ABC Rabbit IgG kit, Vector Lab, Burlingame, CA). Slides were counterstained with hematoxylin. Negative controls were run for every assay.

Immunoreactivity was quantified by computerized densitometry using the ImagePro Plus, version 5.1 software (Media Cybernetics, Silver Spring, MD) coupled to an Olympus BHS microscope equipped with a VCC video camera as described previously (Braga et al, 2008). In brief, after images are properly calibrated for background lighting, optical density per cell (OD/AREA) results are proportional to the unweighted average optical density, which is used to determine the concentration of immunoreactive antigen.

2.6. Western blotting

Western blotting was performed using muscle lysates as described previously (Braga et al, 2008; Brown et al, 2009; Sinha-Hikim et al, 2007). In brief, proteins (50–80 µg) were separated on a 4–12% SDS-polyacrylamide gel with MES or MOPS buffer purchased from Invitrogen (Carlsbad, CA, USA) at 200V. Gel was transferred on an Immuno-blot PVDF Membrane (Bio-Rad, Hercules, CA) overnight at 4°C. Membranes were blocked in blocking solution (0.3% Tween 20 in Tris-buffered saline and 10% nonfat dry milk) for 1 h at room temperature then probed using mouse monoclonal phospho-JNK, which detects JNK only when phosphorylated at threonine 183 and tyrosine 185 (1:200; Santa Cruz Biotechnology) and iNOS (1:200; Santa Cruz Biotechnology), and rabbit polyclonal BCL-2 (1:200), caspase 3 and caspase 9 (1: 200; Cell Signaling Technology, Inc., Beverly, MA) for 1 h at room temperature or overnight at 4°C with constant shaking. Following 3 X 10-min washes in TBS-T buffer, membranes were then incubated in anti-rabbit (Amersham Biosciences, Piscataway, NJ, USA) or anti-mouse IgG-HRP (Santa Cruz Biotechnology) secondary antibodies at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Hyper film ECL. The membranes were stripped and reprobed with a rabbit polyclonal GAPDH (1:2000) for normalization of the loading.

2.7. Statistical analysis

Statistical analysis was performed using the SigmaStat 2.0 Program (Jandel Cooperation, San Rafael, CA). Results were tested for statistical significance using the Tukey or Student-Newman-Keuls Method test after one-way ANOVA. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Apoptosis

Figure 1 shows the effects of NAC and a novel GSH precursor F1 in preventing spermine-induced VSMC apoptosis. Apoptosis was detected by TUNEL assay and nuclei were visualized by DAPI. Little or no apoptosis was observed in control human VSMC (panels I and V). Exposure of VSMC to 15 µM spermine resulted in activation of apoptosis (panels II and VI). Apoptosis was detected as early as 8h after spermine treatment and peaked at 24h of exposure. Treatments with both NAC (panels III and VII) and F1 (panels IV and VIII) attenuated spermine-induced VSMC apoptosis. The apoptotic nature of cell death was further confirmed by electron microscopy (Sinha-Hikim et al, submitted for publication and no overlap with this manuscript). The effects of graded doses (50–500 µg/ml) of each antioxidant on spermine-induced VSMC apoptosis (expressed as % of apoptotic cells) are summarized in Fig. 2. At 24 hours after spermine exposure the percentage of apoptotic cells ranged from 25.5 to 31.9%. Co-treatment with NAC and F1 at graded doses (50 µg/ml–500 µg/ml) attenuated spermine-induced apoptosis. However, only F1 at 200 and 500 µg/ml reduced spermine-induced VSMC apoptosis further, with values similar to control levels.

3.2. JNK activation and induction of iNOS

Activation of JNK, as evidenced by a significant ($P < 0.05$) increase (2.2-fold) in phospho-JNK levels, was detected by an EIA assay 24 h after spermine treatment (Fig. 3A). Spermine-induced activation of JNK was significantly prevented by both NAC and F1 at a dose of 200 µg/ml (Fig. 3A). Effects of these antioxidants on spermine-induced JNK activation were further substantiated by western blotting. As shown in Figure 3B, human VSMC treated with spermine had increased phospho-JNK levels. Co-treatment with either NAC or F1 effectively prevented such spermine-induced increase in phospho-JNK levels.

Because activation of JNK is known to induce iNOS (Lahti et al, 2006; Weston and Davis, 2002;), which plays an important role in induction of apoptosis in various cell systems (Liao et al, 2006; Pervin et al, 2003; Vera et al, 2006), we further examined the role of iNOS in spermine-induced VSMC apoptosis and its prevention by antioxidants. As shown in Figure 3B, western blot analysis revealed an increase in iNOS levels in VSMC after spermine treatment and that could be effectively suppressed by co-treatment with F1, in particular. Effects of these antioxidants on spermine-induced up-regulation of iNOS were further substantiated by immunocytochemistry. Compared with controls, a marked increase in iNOS immunoreactivity was noted in these cells as early as 8 h after spermine treatment (Fig. 3C). A redistribution of iNOS from a cytoplasmic to nuclear localization was evident in some cells after induction of apoptosis (Figure 3C). Co-treatment with 200 μ g/ml F1, but not NAC, effectively prevented iNOS induction in spermine-exposed cells. These findings were further corroborated by image analysis (Fig. 3D).

3.3. BAX/BCL-2 ratio

Because the ratio of pro-apoptotic and anti-apoptotic BCL-2 family members such as BAX/BCL-2 constitutes a rheostat that sets the thresholds for susceptibility to apoptosis (Danial and Korsmeyer 2004), we examined the expression profile of BAX and BCL-2 by immunoblotting (Fig. 4A). Western blot analysis showed no obvious alterations of BAX levels after spermine treatment. In contrast, we found a marked decrease in BCL-2 levels after spermine treatment. Addition of both NAC and F1 effectively restored spermine-induced decrease in BCL-2 expression. Changes in BCL-2 expression were further ascertained by immunocytochemistry. As shown in Fig. 4B, we found a decrease in BCL-2 expression as early as 8h of spermine exposure, and that could be effectively attenuated by co-treatment with either NAC or F1. This was further corroborated by image analysis of changes in the staining intensity (Fig. 4C).

3.4. Cytosolic translocation of cytochrome C and activation of caspase 9 and caspase 3

We examined the role of NAC and F1 in preventing spermine-induced cytochrome c release (Fig. 5). Control VSMC exhibited a strong punctate perinuclear staining of cytosolic cytochrome indicative of its mitochondrial localization. Spermine-induced apoptotic cells exhibited diffuse and lesser cytoplasmic staining, which is consistent with its translocation from mitochondria to cytoplasm (Vera et al, 2004). Consistent with an earlier report (Nur-E-Kamal et al, 2004), many apoptotic cells, identified by cell shrinkage and chromatin condensation, also displayed cytochrome c immunoreactivity in the nuclei. Concomitant administration of NAC and F1 were able to prevent such spermine-induced cytochrome c release as visualized by immunocytochemistry.

Because the release of cytochrome c from mitochondria triggers caspase activation, we then examined the activation of the initiator caspase 9 and caspase 3 (Fig. 6). We found activation of caspase 9, as evidenced by immunocytochemical staining of active caspase 9 (Fig. 6A) as well as by immunoblotting (Fig. 6B) in VSMCs after spermine treatment. Caspase 3 was also activated as evidenced by immunoblotting (Fig. 6B). Notably, co-treatment with F1 but not NAC fully prevented spermine-induced activation of both caspases in these cells (Fig. 6B).

4. Discussion

This is the first report to our knowledge that demonstrates: 1) spermine induces VSMC apoptosis through activation of JNK and NO-mediated intrinsic pathway signaling; 2) GSH precursors such as F1 and to a lesser extent NAC can prevent spermine-induced VSMC apoptosis; and 3) the protection conferred by these antioxidants involved suppression of

JNK activation, iNOS induction, and, in turn, inhibition of the intrinsic pathway signaling. However, unlike F1, we found that NAC reduces cell death in a dose response manner. Treatment with the highest dose (500 $\mu\text{g/ml}$) of NAC was found to be least effective in preventing spermine-induced VSMC apoptosis. The reason for limited effectiveness of 500 $\mu\text{g/ml}$ of NAC in preventing cell death remains unknown. However, one possibility may relate to the toxic effects of NAC when given at high dosage. This is consistent with earlier works indicating that while NAC at moderate doses is effective in attenuating iohexol-induced apoptosis of renal proximal tubular epithelial cells, it becomes toxic when given at higher doses (Gong et al, 2010).

Oxidative stress contributes to apoptosis in various cell types (Meurette et al, 2005; Nadaeri et al, 2003; Prasad et al, 2006). Spermine-mediated apoptosis triggered by oxidative stress has also been demonstrated in variety of cell systems, including ischemia-induced apoptosis of cardiac myoblasts (Maccarrone et al, 2001; Tantini et al, 2005; Takano et al, 2005). Emerging evidence now suggest that oxidative stress can promote JNK activation (Ogino et al, 2009; Osto et al, 2008; Wei et al, 2008), which constitutes a critical component of apoptotic signaling in various cells, including muscle cells (Braga et al, 2008; Nadeau et al, 2009; Philpott and Facci, 2008; Sinha-Hikim et al, 2007; Tamango et al, 2003). Thus, the signal for JNK activation most likely emanates from spermine-induced generation of oxidative stress. Consistent with this notion, in a recent study, we showed that spermine induces oxidative stress in VSMC, as evidenced by a reduction in intracellular glutathione levels coupled with an increase in the levels of 4-HNE protein adduct (Sinha-Hikim et al, submitted for publication).

The downstream signaling events that couple JNK activation with VSMC death have not been previously identified. One possibility is that JNK could induce apoptosis through induction of iNOS, resulting in activation of the cytochrome c mediated-death pathway. Indeed, NO production thorough upregulation of iNOS has been reported to be triggered by JNK (Lahti et al, 2006; Weston and Davis, 2002). Available evidence also suggests that both JNK (Lei et al, 2002; Sinha-Hikim et al, 2007; Tournier et al, 2000; Tsuruta et al, 2004) as well as iNOS (Liao et al, 2006; Sinha-Hikim et al, 2007; Vera et al, 2006) can induce apoptosis in various cell systems through activation of the cytochrome c-mediated death pathway. Indeed, in the present study, we found induction of iNOS in VSMCs after spermine treatment, which was further associated with perturbation of the BAX/BCL-2 rheostat, cytosolic translocation of cytochrome c and activation of the initiator caspase 9. This is consistent with earlier works indicating the involvement of the mitochondria-dependent intrinsic pathway signaling in some cell lines after spermine exposure (Maccarrone et al, 2001; Schiller et al, 2005). Together, these data indicate that JNK and NO-mediated intrinsic pathway signaling plays an important role in spermine-induced VSMC apoptosis.

Glutathione is widely considered the cell's most important antioxidant that neutralizes free radicals produced in response to various agents and oxidative damages (Sen et al, 1997; White et al, 1994; Wu et al, 2004). GSH is synthesized from cysteine (Cys), glycine, and glutamate, with Cys availability often being the rate-limiting factor (Miller et al, 2002). The predominant form of Cys in the plasma is the oxidized disulfide form, cystine (CySS), and the Cys/CySS redox state can modulate multiple cellular events (Jonas et al, 2002; Ramirez et al, 2007), including apoptosis (Go and Jones, 2005; Valko et al, 2006). In fact, early depletion of GSH is a common feature of apoptosis triggered by a variety of stimuli (reviewed in Franco and Cidlowski, 2009). However, when administered orally, glutathione cannot be taken up by mammalian cells since in the gastrointestinal tract it is largely converted into constituent amino acids (including the highly oxidizable L-cysteine) which cannot be re-constituted into glutathione extracellularly as its re-assimilation requires two

cytosolic, ATP-dependent enzymes. Intravenous glutathione administration is expensive, short-lived, and impractical, as the transient rise in plasma concentration is largely ineffective in improving intracellular glutathione activity, where much of its redox actions take place. However, a glutathione precursor, with cystine as the physiologic cysteine carrier, could create a more optimal Cys/CySS redox state, and a greater cellular delivery of substrate to attenuate oxidative stress to a greater degree than traditional glutathione precursors, which despite showing promise (Drager et al, 2004; Lopez et al, 1998), have not demonstrated consistent clinical efficacy (Kshirsagar et al, 2004; Miner et al, 2004; Nallmothu et al, 2004).

Here we show that supplementation with F1, a potent amino-acid premix fortified with selenium, was more effective in preventing spermine-induced VSMC apoptosis than NAC alone. The two pathways through which F1 are posited to exert its enhanced affect are: 1) the use of cystine, rather than the more highly oxidizable cysteine as a GSH precursor to enhance intracellular GSH; and 2) the addition of selenium, as glutathione peroxidase (GPX) is selenium-based and the key enzyme for reduction of lipoperoxides and conversion of hydrogen peroxide to water using reduced glutathione as substrate

In summary, the present study demonstrated that the reputed uremic toxin, spermine can promote VSMC apoptosis and that spermine-induced apoptosis can be attenuated by glutathione-based antioxidant compounds. The study further revealed that attenuation of spermine-induced VSMC apoptosis by the given compounds is mediated by their ability to suppress JNK and NO-mediated intrinsic pathway signaling. This effect was most robust with the addition of F1, a cystine-based GSH precursor supplemented with selenium. Given the limited effectiveness of GSH when given orally or intravenously in restoring its intracellular stores, the efficacy of FI demonstrated here points to its potential as a promising agent for treatment of oxidative stress-induced medical conditions such as CKD and CKD related atherosclerosis.

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Abbreviations

CKD	chronic kidney disease
NAC	N-acetylcysteine
GSH	glutathione
VSMC	vascular smooth muscle cell
JNK	c-Jun-NH ₂ -terminal kinase
iNOS	inducible nitric oxide synthase
CVD	cardiovascular disease
ROS	reactive oxygen species
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling

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Fig. 1. DAPI (I–IV) and TUNEL (V–VIII) show effective suppression of spermine-induced VSMC apoptosis by antioxidant treatments. Compared to control, where no apoptosis is detected, spermine results in activation of VSMC apoptosis (arrow) and which could be effectively prevented by concomitant treatments with both NAC and F1. Nuclei are visualized by DAPI staining. Scale bar = 50 μ m.

Fig. 2. Effect of graded doses (50–500 µg/ml) of antioxidants on spermine-induced VSMC apoptosis after 24 h of treatment. Co-treatment with 200 or 500 µg/ml F1 but not NAC fully ($P < 0.001$) suppressed spermine-induced VSMC apoptosis. Values are mean $\pm$ SEM. Means with unlike superscripts differ significantly.

Fig. 3.

Activation of JNK, as measured by Titer Enzyme EIA Kit (A) and by western blotting (B). (A) JNK activation, as evidenced by a significant ($P < 0.05$) increase in phospho-JNK levels, is detected in VSMC lysates 24 h after exposure to spermine (sp). Co-treatments with both NAC and F1 significantly ($P < 0.05$) prevents spermine-induced activation of JNK. Values are mean $\pm$ SEM. Means with unlike superscripts differ significantly. (B) Representative western blots of cell lysates from control (C), spermine (Sp), spermine + NAC, and spermine + F1 groups show effective suppression of spermine-induced activation of JNK by both antioxidants, while induction of iNOS by spermine is only blocked by F1. GAPDH in the immunoblot is shown as a loading control. (C) Immunocytochemical changes in the iNOS expression in VSMCs in various treatment groups. Compared with control, an increase in iNOS immunoreactivity (arrow) is noted in these cells after spermine treatment.

Concomitant treatments with F1 but not NAC prevent such spermine-induced increase in iNOS expression in these cells. Scale bar = 50 μ m. (D) Computerized densitometric analysis shows a significant increase in iNOS immunoreactivity in VSMCs after spermine treatment compared to that of controls. Co-treatment with F1 significantly ($P<0.001$) prevents such spermine-induced increase in iNOS expression. Values are mean $\pm$ SEM. Means with unlike superscripts differ significantly.

Fig. 4.

(A) Western blot analysis of BAX and BCL-2 in VSMC lysates from control and various treatments groups show no obvious alterations of BAX levels, whereas BCL-2 levels appear to be markedly decreased after spermine treatment. Addition of both NAC and F1 effectively restored spermine-induced decrease in BCL-2 expression. GAPDH in the immunoblot is shown as a loading control. (B) Immunocytochemical changes in the expression of BCL-2 in VSMCs in various treatment groups. Exposure of VSMC to spermine results in a decrease in BCL-2 immunostaining after exposure to spermine. Addition of F1 to spermine fully restored such spermine-induced decrease in BCL-2 expression in these cells. Scale bar = 50 μ m. (C) Computerized densitometric analysis shows a significant decrease in BCL-2 immunoreactivity in VSMCs after spermine treatment compared to that of controls. Co-treatments with F1 but not NAC significantly ($P < 0.05$) restores such spermine-induced depletion in BCL-2 expression. Values are mean $\pm$ SEM. Means with unlike superscripts differ significantly.

Fig. 5.

Visualization of cytochrome c release after activation of apoptosis and its prevention by antioxidants by immunocytochemistry. Control VSMCs exhibit a strong punctate perinuclear staining of cytosolic cytochrome (asterisk) indicative of its mitochondrial localization. After apoptosis induction, these cells show mostly diffuse cytoplasmic staining, which is consistent with its translocation from mitochondria to cytoplasm. Many apoptotic cells, identified by cell shrinkage and chromatin condensation, also display cytochrome c immunoreactivity in the nuclei (arrow). Concomitant administration of F1 in particular, is able to prevent such spermine-induced cytochrome c release. Scale bar = 25 μ m.

Fig. 6.

(A) Activation of caspase 9, as evidenced by immunocytochemical staining of active caspase 9 in VSMCs after spermine treatment, which could be effectively prevented by either NAC or F1. Compared with control, where little or no expression of active caspase 9 is detected, a marked increase in active caspase 9 immunoreactivity is noted in these cells 24 h after spermine treatment. Co-treatment with either NAC or F1 effectively prevents such spermine-induced activation of caspase 9. Scale bar = 50 μ m. (B) Western blot analysis shows that spermine-induced VSMC apoptosis is associated with activation of caspase 9 and caspase 3. Notably, co-treatment with F1 but not NAC fully prevents spermine-induced activation of caspase 9 and caspase 3 in these cells.