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Effects of a novel cystine-based glutathione precursor on oxidative stress in vascular smooth muscle cells

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Sinha-Hikim I, Shen R, Lee WN, Crum A, Vaziri ND, Norris KC. Effects of a novel cystine-based glutathione precursor on oxidative stress in vascular smooth muscle cells. *Am J Physiol Cell Physiol* 299: C638–C642, 2010. First published June 30, 2010; doi:10.1152/ajpcell.00434.2009.—Chronic kidney disease (CKD) is associated with accelerated atherosclerosis and cardiovascular disease, which is largely mediated by oxidative stress. We investigated the effect of three glutathione (GSH) precursors: *N*-acetyl-cysteine (NAC), cystine as the physiological carrier of cysteine in GSH with added selenomethionine (F1), and NAC fortified with selenomethionine (F2) on oxidative stress induced by spermine (a uremic toxin) in cultured human aortic vascular smooth muscle cells (VSMC). VSMC were exposed to spermine (15 μM) with or without the given antioxidants (dose 50, 100, 200, and 500 μg/ml) or vehicle (control) and assessed for intracellular GSH levels, 4-hydroxy-trans-2-nonenal (4-HNE), and incorporation of ^{13}C from glucose into alanine and protein. Spermine exposure reduced intracellular GSH levels, increased 4-HNE, and impaired glucose metabolism through reduction in pyruvate generation and/or transamination. Treatment with NAC had no effect on intracellular glutathione level. In contrast, F1 maintained intracellular GSH at control levels at all four doses. Subsequent studies performed with 200 μg/ml of F1, F2, or NAC (optimal dose) revealed normalization of 4-HNE, whereas restoration of ^{13}C from glucose to alanine or protein to control values was only noted in the F1 group. Spermine-induced alterations in VSMC ultrastructure were prevented in ~90% of cells treated with F1 but only ~50% of cells treated with either NAC or F2. In conclusion, F1 was more effective than NAC or F2 in ameliorating spermine-induced reduction in intracellular GSH levels and cellular alterations in VSMC. The cystine-based GSH precursor (F1) is a promising antioxidant, and further studies are needed to examine the effect of this compound in preventing CKD-associated vascular disease.

spermine; antioxidants; chronic kidney disease

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 mechanisms through which CKD promotes CVD include, but are not limited to, hemodynamic (e.g., increased blood pressure), and nonhemodynamic events including oxidative stress, inflammation, dyslipidemia, and accelerated apoptosis (6, 7, 11, 12, 19, 30, 32).

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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 (11). In addition, oxidative stress contributes to macrophage and vascular smooth muscle cell (VSMC) apoptosis, plaque instability, rupture, and thrombosis (1, 6, 7). Spermine, a polyamine product, is a reputed uremic toxin that has been implicated as a potential mediator of a number of the CKD-associated clinical abnormalities (22). 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 (23).

Normally cells are equipped with an elaborate defense system consisting of numerous antioxidant enzymes and scavenger molecules that 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 (L-γ-glutamyl-L-cysteinyl-glycine; GSH), which is critical for the maintenance of the cellular redox balance (9, 33). In fact, selective inhibition of enzymes of the glutathione redox cycle heightens the susceptibility to ROS-mediated cell injury (8) and hypertension (31). 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 GSH peroxidase (9, 33).

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 (9, 10, 31, 33). CKD results in depletion of reduced glutathione (GSH) and other thiols and marked reduction of GSH/oxidized glutathione (GS-SG) ratio (7). *N*-acetyl-cysteine (NAC), a glutathione precursor has shown promise as an effective antioxidant (8, 17, 24), although clinical findings have been inconsistent (15, 20, 21). Given the potential role of oxidative stress in CKD-related accelerated CVD and the central role of GSH in cellular defense against ROS-mediated injury, we hypothesized that strategies aimed at GSH repletion may attenuate spermine-induced oxidative stress. To this end we assessed the efficacy of NAC and two novel GSH precursors on preventing oxidative stress in spermine-exposed cultured human VSMC as a model of CKD-related vascular disease.

MATERIALS AND METHODS

Antioxidants. We used NAC (Sigma-Aldrich), FT061452/RE39734 or F1, a GSH precursor with cystine replacing cysteine and selenome-

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thionine added (ProImmune, Rhinebeck, NY), and FT061453/RE39734 or F2, NAC fortified with selenomethionine (ProImmune, Rhinebeck, NY). The F1 formulation (per 500 mg) contains 99.68 mg L-cystine, 199.39 mg glycine, 1.54 mg selenomethionine, and 199.39 mg L-glutamine. The F2 formulation (per 500 mg) contains 166.14 mg NAC, 166.14 mg glycine, 1.54 mg selenomethionine, and 166.14 mg L-glutamine.

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, St. Louis, MO); *2*) spermine + NAC (Sigma-Aldrich); *3*) spermine + F1; *4*) spermine + F2; and 5) control. The 15 μM concentration of spermine used in the present study was based on the results of our preliminary dose-response study analyses. NAC, F1, and F2 were tested in the 50–500 μg/ml dose range, and we found 200 μg/ml of antioxidants were optimal for studying metabolic and signal transduction pathways.

For metabolic studies, on the day of experiment the cell culture medium was replaced by glucose-free DMEM medium (GIBCO-BRL, Invitrogen, Carlsbad, CA), supplemented with 1,2-[$^{13}C_2$]glucose (Cambridge Isotope, Andover, MA) at the concentration of 1 g/l for 24 h. At the end of the incubations, cells were separated by centrifugation (1,500 rpm for 5 min), and incubation medium and cell pellets were obtained for tracer-based metabolic studies in conjunction with gas chromatography/mass spectrometry (GC/MS) analysis (3, 13).

Reduced GSH assay. Reduced GSH levels were measured using a commercial kit according to the manufacture's protocol (BIOXYTECH GSH/GSSG-412 assay kit; OXISResearch, a division of Oxis Health Product, Portland, OR). This assay, using different sample preparations, measures both reduced (GSH) and oxidized (GSSG) concentrations and the GSH-to-GSSG ratio. The omission or addition of 1-methyl-2-vinlylpyridinium trifluromethanesulfonate allows the measurement of GSH and GSSG, respectively. However, the measurement of GSH-to-GSSG ratio was not possible in VSMC lysates due to extremely low value (near noise levels) of GSSG. Briefly, the assay measures the total (reduced and oxidized) GSH concentrations using GSSG reductase, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and nicotinamide adenine dinucleotide phosphate (NADPH) recycling procedure. The assays uses GSSG reductase and NADPH to recycle a GSH-TNB adduct to produce thio-bis-(2-nitrobenzoic acid) (TNB), a spectrophotometrically detectable product at 412 nm. The change in absorbance at 412 nm is a linear function of the GSH concentration in the reaction mixture. This kit is highly specific for GSH (100%), with negligible specificity to *N*-acetyl cysteine (0.038%), cysteine (0.16%), and cystine (0.14%). The precision of measurement is high with an intra-assay CV% of 0.96 and interassay CV% of 3.11.

Measurements of 4-hydroxy-trans-2-nonenal. Further evaluation of oxidative stress was achieved by measuring 4-hydroxy-trans-2-nonenal (4-HNE), a biomarker of oxidative stress (14, 28), using an ELISA kit (Cell Biolabs, San Diego, CA). In brief, 4-HNE protein adducts present in the sample or standard are probed with the primary 4-HNE antibody, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The 4-HNE protein adduct content in the unknown sample is determined by comparing with the standard curve that is prepared from predetermined HNE-BSA standards.

Transmission electron microscopy. To assess restoration of overall cellular morphology of spermine-treated VSMC by these antioxidants, we performed electron microscopy. The cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.05 M caccodylate buffer (pH 7.4) (Polysciences, Warrington, PA) for 3 h at room temperature, postfixed with 1% osmium tetroxide, dehydrated with graded series of ethanol, and embedded in Epon as described previously (25, 26). Thin sections from selected tissue blocks containing cells from each group were sectioned with an LKB ultramichrotome, stained with uranyl acetate, and examined in a Hitachi 600 electron microscope (Hitachi, Indianapolis, IN). Micrographs were taken by an observer who was unaware of the treatment assignment. From each treatment group 40 micrographs were selected for ultrastructural analysis.

Statistical analyses. Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel, 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 $P < 0.05$.

RESULTS

Intracellular GSH levels. Data are shown in Fig. 1. Exposure of human VSMC to 15 μM spermine resulted in marked reduction of the intracellular total GSH levels (which are

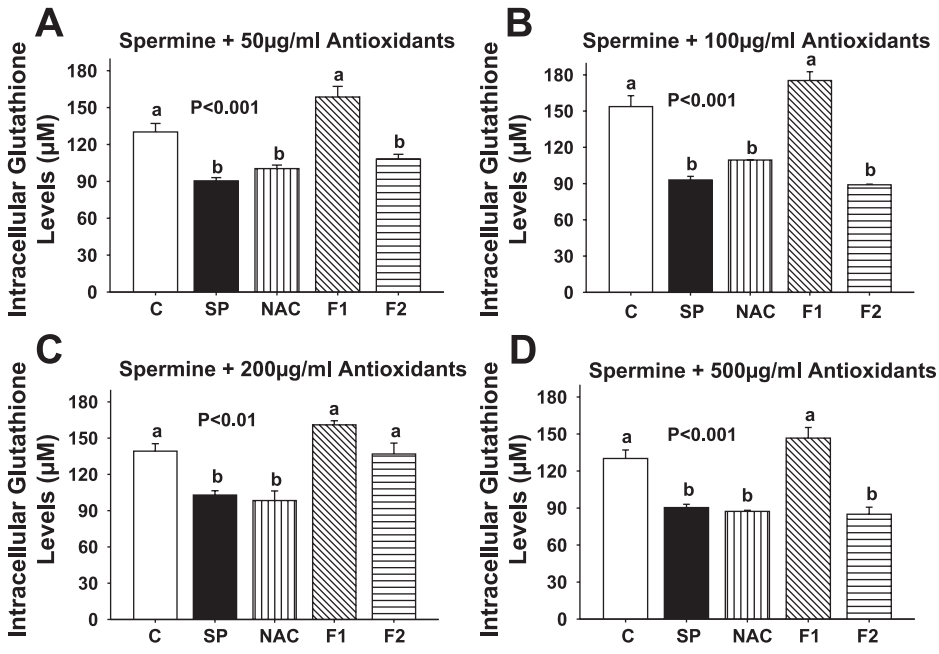

Fig. 1. Exposure of human vascular smooth muscle cells (VSMC) to spermine (SP) significantly reduces intracellular total glutathione (GSH) levels and which can be fully prevented by F1 at all dose levels. F2 is only effective at 200 μg/ml dose levels in restoring SP-induced depletion of intracellular GSH levels. However, *N*-acetyl-cysteine (NAC) has no effect on SP-induced depletion of intracellular GSH levels. Values are means ± SE. Means with unlike superscripts differ significantly. Data are based on three replicates from 3 separate experiments. C, control; F1 and F2, see MATERIALS AND METHODS.

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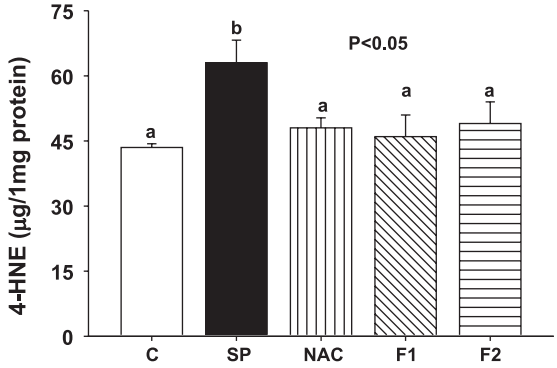

Fig. 2. ELISA assay shows increased levels 4-hydroxy-trans-2-nonenal (4-HNE) in VSMC lysates 24 h after exposure to spermine. Cotreatments with all three antioxidants (NAC, F1, and F2) significantly ($P < 0.05$) prevent SP-induced increase in 4-HNE levels. Values are means ± SE. Means with unlike superscripts differ significantly. Data are based on three replicates from 3 separate experiments.

essentially equivalent to GSH levels since GSSH was undetectable) within 24 h compared with control ($P < 0.001$). Treatment of spermine-exposed cells with NAC had no effect on intracellular GSH levels, whereas treatment of spermine-exposed cells with F1 (cystine-based GSH precursor with selenomethionine) achieved intracellular GSH levels similar to control cells at all doses, 50, 100, 200, and 500 μg/ml. By contrast, treatment with F2 prevented depletion of intracellular GSH levels only at 200 μg/ml dose levels.

4-HNE protein adducts. We next examined another biomarker of oxidative stress, the lipid peroxidation marker 4-HNE (14, 28). As shown in Fig. 2, human VSMC treated with 15 μM spermine for 24 h had significantly ($P < 0.05$) higher levels of 4-HNE protein adducts. Cotreatment with 200 μg/ml of all three antioxidants completely prevented spermine-induced increase in 4-HNE protein adducts.

VSMC ultrastructure. We performed electron microscopy to evaluate cellular morphology. Control VSMC exhibited nuclei with smooth contours, evenly dispersed chromatins, and contained abundant cytoplasmic organelles (Fig. 3*A*). After exposure to spermine, these cells appeared shrunken and exhibited chromatin condensation and fragmentation, apoptotic bodies, cytoplasmic vacuolization, and reduction in cellular organelles (Fig. 3*B*). The morphology of a majority (~90%) of spermine exposed VSMC cotreated with 200 μg/ml F1 was similar to control (Fig. 3*C*). By contrast, ~50% of spermine exposed VSMC cotreated with 200 μg/ml of either NAC or F2 exhibited apoptotic morphology (Fig. 3*D*).

Metabolic studies. Glycolysis of [1, 2,$^{13}C_2$]-glucose produces [2,3$^{13}C_2$]lactate/pyruvate, which becomes [2,3$^{13}C_2$] alanine (M+2 alanine) by transamination. Mass spectral data revealed that incorporation of ^{13}C into alanine, and protein was significantly reduced following treatment with

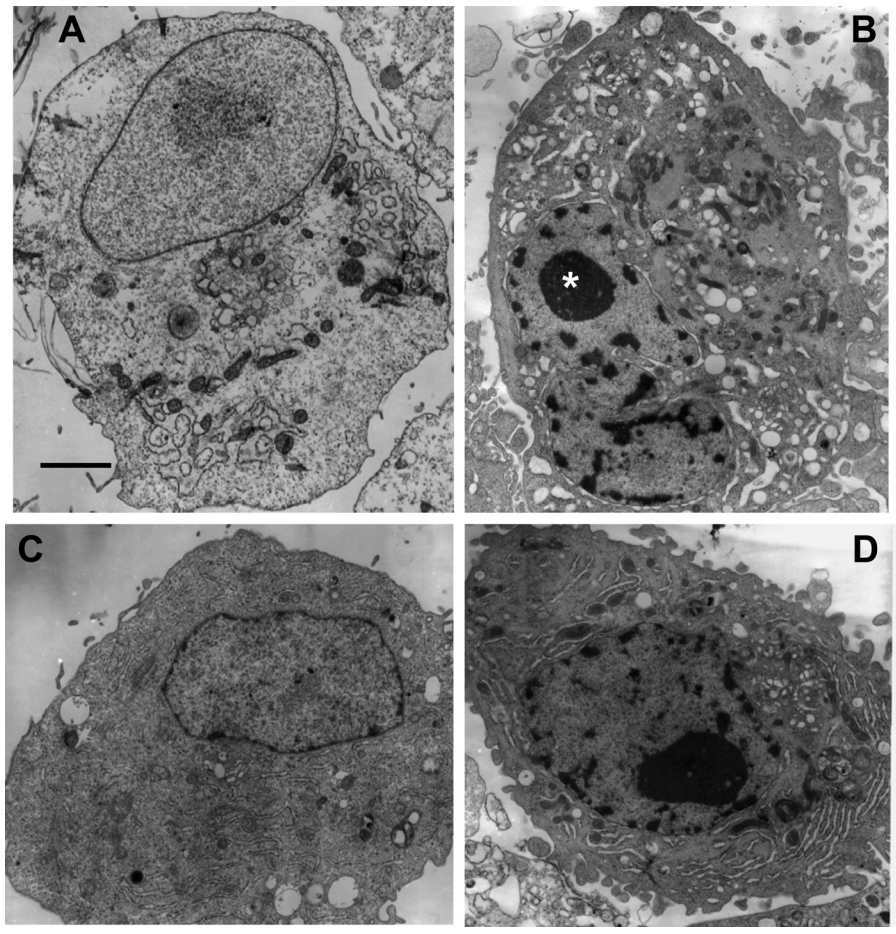

Fig. 3. *A*: representative control VSMC shows normal ultrastructure, characterized by nucleus with smooth contour, evenly dispersed chromatin, and abundant cytoplasmic organelles. After induction of apoptosis by spermine, these cells exhibit characteristic apoptotic changes, including chromatin condensation and fragmentation, formation of apoptotic bodies (asterisk), cytoplasmic vacuolization, and reduction in cellular organelles (*B*). The overall morphology of the majority of rescued VSMC after F1 treatment is essentially similar to that of controls (*C*). By contrast, ~50% of the cells in spermine + NAC or F2-treated group exhibit apoptotic morphology (*D*). Scale bar = 2.5 μm.

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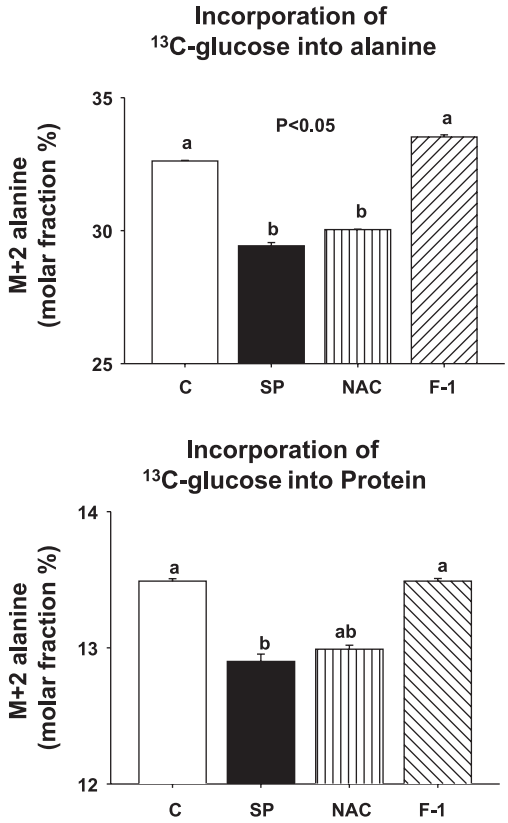

Fig. 4. Changes in incorporation of ^{13}C from glucose into alanine (*top*) and into protein (*bottom*) in SP, SP + NAC (NAC), and SP + F1 (F1) groups. Values are means ± SE. Means with unlike superscripts differ significantly ($P < 0.05$).

spermine (Fig. 4). Therefore, spermine significantly reduces pyruvate generation and/or transamination, pathways responsible for glucose utilization for protein synthesis, in vascular smooth muscle cells. As shown in Fig. 4, spermine-exposed VSMC treated with 200 μg/ml F1 retained the glycolytic and transamination function and the incorporation of ^{13}C in alanine commensurate with control VSMC. The addition of NAC to spermine did not restore incorporation of ^{13}C from glucose to alanine (Fig. 4).

DISCUSSION

To our knowledge, this is the first study to examine the effects of a cystine-based glutathione precursor with added selenomethionine on oxidative stress. Here we show that spermine treatment induces oxidative stress, as evidenced by a reduction in intracellular GSH levels coupled with an increase in the levels of 4-HNE protein adducts in VSMC. Our findings illustrate the ability of GSH precursor to attenuate spermine-induced oxidative stress by increasing cellular GSH levels and preventing oxidative stress-mediated reduction in nonessential amino acid synthesis from glucose for protein anabolism.

Oxidative stress has been implicated in the pathogenesis of CKD, cardiovascular diseases, and atherosclerosis, in addition to many other diseases (11, 16, 24, 30, 32). Our findings are consistent with previous studies assessing the antioxidant properties of NAC, which has been shown to reduce superoxide production leading to potentially favorable clinical effects such as enhancing endothelium-dependent vasorelaxation (17) and reducing atherosclerosis (24). At the level of the kidney NAC might protect against contrast nephropathy (8), although the results of the clinical studies have been inconsistent (15, 20, 21). Our findings are consistent with prior studies indicating involvement of oxidative stress in spermine-induced cell death in a variety of cell systems including microglial cells, primary human blood cells, and various tumor cell lines (18, 23, 29). Takano et al. (29) reported that antioxidant agents such as GSH and NAC prevented spermine cytotoxicity in microglial cells supporting spermine-induced cell damage via oxidative stress. The positive effects of F1 on intracellular GSH and select markers of oxidative stress in spermine-exposed VSMC employed in our study were similar or superior to NAC.

The present study was not designed to elucidate the precise mechanisms by which spermine decreases GSH levels in VSMC. However, based on available information, we speculate that depletion of intracellular GSH could occur through extrusion of reduced GSH and/or decreased GSH biosynthesis (reviewed in Ref 4). Depletion of GSH is an early hallmark of apoptosis (4, 5). Whereas it is still unclear how GSH depletion induces apoptosis in VSMC after spermine treatment, depletion of GSH has been shown to involve c-Jun-NH_2-terminal kinase (JNK) activation, dissipation of mitochondrial membrane potential, release of mitochondrial cytochrome c into the cytosol, and activation of downstream caspases (4, 5). Consistent with this model, in a recent study, we have shown that indeed spermine-induced VSMC apoptosis is associated with activation of JNK, perturbation of the BAX/BCL-2 rheostat, cytochrome c release from mitochondria into the cytosol, and activation of caspase 9 and caspase 3 (27). It is possible that depletion of intracellular GSH could sensitize VSMC to spermine-induced apoptosis by triggering the JNK-mediated mitochondria-dependent apoptotic pathway. Thus restoration of the intracellular GSH levels is critical for F1-induced protection of VSMC against spermine injury. Data reported herein further indicate that while cotreatment with 200 μg/ml of all three antioxidants completely attenuated spermine-induced increase in 4-HNE levels, only F1 at all doses and F2 at 200 μg/ml are effective in restoring the intracellular GSH levels in spermine-treated VSMC.

The observation of decreased alanine labeling from [^{13}C]glucose with spermine treatment suggests impairment of glucose metabolism through reduction in pyruvate generation and/or transamination by spermine, which remained normal with F1 treatment. The exact mechanism through which spermine and select antioxidants interact with glucose metabolism remains to be investigated. In addition, the protection of VSMC ultrastructure with F1 suggests that spermine-induced structural alterations in VSMC can be prevented by effective antioxidant treatment.

The underlying mechanisms by which F1 prevents the adverse effects of spermine on VSMC are not known. We are, however, intrigued by the observation that, compared with F1, F2 (NAC fortified with same amount of selenomethionine-like F1) is less effective in ameliorating spermine-induced reduction in GSH levels and perturbation in cellular morphology. Thus it is conceivable that cystine replacing cysteine in F1 formulation may play an important role in F1-mediated protection of VSMC against spermine-induced injury. This notion is supported by another line of evidence showing that F1 is

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more effective than NAC in preventing spermine-induced VSMC apoptosis through suppression of JNK and nitric oxide-mediated intrinsic pathway signaling (27).

In summary, the present study demonstrates that a novel GSH precursor was highly effective in reducing oxidative stress and restoring protein metabolism in spermine-treated VSMC. These findings support the potential of this precursor as a therapeutic intervention and the need for additional studies to clarify the mechanistic pathways and efficacy in the prevention/treatment of oxidative stress-induced medical conditions such as CKD and CKD-related atherosclerosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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