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Homocysteine induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite

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Abstract

Homocysteine (HCY) is toxic on blood vessels, but a potential direct toxicity of HCY on the heart is unknown. We addressed this issue by exposing H9C2 cardiomyocytes to HCY (0.1–5 mM) for up to 6 h. At these concentrations, HCY reduced cell viability, induced necrosis and apoptosis and triggered the cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP). This was associated with the intracellular generation of the potent oxidant peroxynitrite. Removing peroxynitrite by the decomposition catalyst FeTPPS considerably reduced LDH release, DNA fragmentation, cleavage of caspase-3 and PARP, and restored normal cell morphology. In additional experiments performed in primary rat ventricular cardiomyocytes, HCY (1 mM, 6 h) activated the phosphorylation of the MAP kinases ERK and JNK, two essential stress signaling kinases regulating myocardial apoptosis, hypertrophy and remodeling. These results provide the first demonstration that HCY kills cardiomyocytes through the generation of peroxynitrite and can activate key signaling cascades in the myocardium.

Keywords

Homocysteine; Oxidative stress; Peroxynitrite; Apoptosis; Necrosis; Cardiomyocyte; MAP kinase; ERK; JNK

Elevated plasma levels of homocysteine (HCY) represent an independent risk factor for cardiovascular diseases [1]. Endothelial injury and dysfunction, related to endothelial oxidative stress [2] and endothelial apoptosis [3] are key pathophysiological mechanisms of HCY-dependent vascular toxicity [1]. Whether HCY may exert similar direct toxic effects on the heart is unknown, but several recent reports raise such a possibility. Experimentally, hyperhomocysteinemia (hHCY) has been associated with disturbed cardiac substrate metabolism [4], and mitochondrial dysfunction [5], as well as adverse cardiac remodeling with increased myocardial stiffness [6,7]. To address the issue of a possible direct toxicity of HCY on cardiac cells, we performed an in vitro study investigating the effects of HCY on cultured H9C2 rat cardiomyocytes, as well as in primary cardiomyocytes isolated from adult rat hearts.

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Material and methods

Cell culture conditions and stimulation with D,L-homocysteine

Rat H9C2 cardiomyocytes were grown as previously mentioned [8]. Cells were exposed to D,L-homocysteine (HCY, Sigma Chemicals, Basel, Switzerland) for 1–6 h at 0.1–5 mM. In experiments using inhibitors, cells were pre-treated for 2 h with 50–250 μM of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis-(4-sulphonatophenyl)-porphyrinato iron (III) (FeT-PPS, Calbiochem, San Diego, CA) or 0.1–3 mM of the NO synthase inhibitor N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, Sigma).

Preparation of adult rat ventricular cardiomyocytes

All procedures were in accordance with the Swiss laws on animal experimentation. Ventricular myocytes were isolated as described [9], with some modifications. Briefly, adult male Wistar rats were anesthetized, their heart was removed and perfused with oxygenated (95% O_2, 5% CO_2) Joklik's medium (Cell Culture Technologies, TI, Switzerland), followed by perfusion (35 min) with a solution of Type II collagenase (80 U/ml) (Worthington Corporation, Lakewood, NJ) containing 200 μM $CaCl_2$. The ventricles were then minced, the cell suspension centrifuged (5 min, 30 g) and the pellet resuspended in M199 medium, supplemented with 20 mM creatine, 0.1 mM ARA-C, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Sigma) and 1% FCS. Cells were plated in a 10-cm dish at 37°C. After 2 h, the medium was changed to 20% FCS and cells were plated in 6-well plates (20,000 cells/cm^2). Adherent cells were used after 8 days for the experiments.

Determination of cell viability and nuclear morphology

Cell viability was assessed by measuring the mitochondrial-dependent reduction of MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, as described [8].

The fluorescent nuclear binding dyes Hoechst 33342 (Calbiochem), which stains DNA blue, and propidium iodide (PI, Sigma), which is impermeable to cells with preserved membranes and stains DNA red, were used to detect necrotic and apoptotic cells, as described [10]. Apoptotic cells were detected by chromatin condensation and nuclear fragmentation, whereas necrotic cells were detected by intact nuclei and positive PI staining. The number of apoptotic and necrotic cells were quantified in 8 random fields at 40× magnification in at least 4 pictures per experimental condition.

Cell death assays: LDH release and apoptotic DNA fragmentation

The release of LDH in the medium was used as an index of necrosis [10], using a commercial kit (Roche Molecular Biochemicals, Basel, Switzerland), whereas the Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Basel, Switzerland) was employed to quantify apoptotic DNA fragmentation [10].

Western blot experiments

Cells were scraped in lysis buffer and the recovered proteins (20 μg) were subjected to standard SDS–PAGE procedure and transfer to nitrocellulose membrane, as described [8]. The membrane was incubated overnight at 4°C with an appropriate dilution of anti-caspase-3 (33 kDa), anti-cleaved caspase-3 (17–19 kDa fragments), anti-PARP-1 (119 kDa), anti-cleaved PARP-1 (89 kDa), anti-phospho-ERK 1/2 (p42/p44 MAP kinase) and anti-ERK1/2, anti-phospho-JNK 1/2 (p46/p54) and anti-JNK1 (all from Cell Signaling, Beverly, MA), and mouse monoclonal anti-nitrotyrosine (Cayman Chemical, Ann Arbor, MI) primary antibodies, followed by incubation for 1 h with a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). The signal was visualized using

enhanced chemiluminescence (ECL, Amersham Biosciences, Otelfingen, Switzerland) and analyzed by densitometry using a Personal Densitometer and TotalLab Software.

Cytological evaluation

Cells cultured on glass coverslips were treated with HCY (1 mM) for 6 h in the absence or in the presence of a 2 h pre-treatment with FeTPPS (250 μM), and were then fixed and stained with Hematoxylineosin for morphological evaluation.

Presentation of data and statistical analysis

All graphs present data as means ± sem of at least three independent experiments. Student's *t* test was used when only two conditions were compared. In experiments with multiple conditions, comparisons between control and HCY treatment were done by ANOVA followed by Dunnett test when appropriate. In experiments using HCY and inhibitors, comparisons were made by ANOVA followed, when appropriate, by Tukey test. A $p < 0.05$ was considered significant.

Results

Homocysteine induces necrotic and apoptotic cell death in H9C2 cardiomyocytes

H9C2 cells treated for 6 h with HCY disclosed a concentration-dependent reduction of cell viability, that was significant at all concentrations of HCY (Fig. 1B). As shown by nuclear staining (Fig. 1A), apoptotic cell death was evident at all concentrations of HCY, while necrotic cell death, indicated by the presence of intact nuclei staining positively for PI, was also clearly induced by HCY treatment, and appeared as the preferential mode of cell demise at the highest concentration of HCY (5 mM). These findings were further confirmed by the quantitative evaluation of apoptotic and necrotic cell death (Fig. 1C), as well as by LDH release (necrosis, Fig. 2A) and apoptotic DNA fragmentation (Fig. 2B), which were significantly increased at all concentrations of HCY.

The proteolytic activation of caspases represents the prototypical signaling mechanism of apoptosis converging to the activation of caspase-3, which cleaves multiple targets, including the nuclear enzyme poly(ADP-ribose) polymerase (PARP). The cleavage of caspase-3 and PARP thus represents a hallmark of apoptosis. As indicated in Fig. 2C and D, HCY induced the cleavage of both PARP and caspase-3 in a concentration- and time-dependent manner.

HCY triggers the generation of peroxynitrite in H9C2 cells and induces the phosphorylation of the MAP kinases ERK and JNK in primary ventricular myocytes

The generation of peroxynitrite was monitored by the formation of 3-nitrotyrosine (3-NT) [11]. As indicated in Fig. 3A, HCY induced the formation of a large band of 3-NT at approximately 65 kDa, that was suppressed by the peroxynitrite decomposition catalyst FeTPPS (Fig. 3B). We previously reported that peroxynitrite is a potent activator of the mitogen-activated protein kinases ERK and JNK in cardiomyocytes [12], which are important signals involved in the mechanisms of cell survival and cell death. Given the ability of HCY to generate peroxynitrite, we sought to determine whether HCY would activate these MAP kinases in primary rat cardiomyocytes. As shown in Fig. 3C, HCY (1 mM) for 6 h resulted in a strong phosphorylation of both ERK1/2 (p42/p44) and JNK 1 (p46), whereas the p54 JNK subunit (JNK2) was only marginally phosphorylated (1.35-fold increase, p = NS). In contrast, nonphosphorylated ERK and JNK were not influenced by HCY (not shown).

The peroxynitrite decomposition catalyst FeTPPS alleviates the cytotoxic effects of HCY in H9C2 cells

The removal of peroxynitrite by FeTPPS significantly alleviated apoptotic DNA fragmentation (Fig. 4A), caspase-3 and PARP cleavage (Fig. 4B and C), as well as the release of LDH triggered by HCY (Fig. 4D). These effects were associated with the restoration of normal cell morphology by FeTPPS (Fig. 4F), pointing to a critical role of peroxynitrite in HCY-mediated toxicity in H9C2 cardiomyocytes. In addition, the NOS inhibitor L-NAME also reduced HCY-induced LDH release (Fig. 4E), implying a role for NOS-derived NO in the formation and toxicity of peroxynitrite upon HCY treatment in these cells.

Discussion

In contrast to the well-described toxicity of HCY on the endothelium, its potential toxicity on the myocardium remains largely unknown. In experimental animals, hyperhomocysteinemia (hHCY) can lead to myocardial fibrosis and functional impairment of the left ventricle, through poorly defined mechanisms [6,7]. Recently, two reports showed that HCY could alter substrate use and the mitochondrial control of oxygen consumption by the myocardium, via a reduced availability of NO, consecutive to its scavenging by superoxide ($O_2^{\cdot -}$), produced by NADPH oxidase in response to HCY [4,5]. We now significantly extent these observations, showing that HCY markedly reduces the viability of H9C2 cardiomyocytes, by triggering both necrotic and apoptotic modes of cell death (Figs. 1 and 2). Although these effects were obtained at relatively high HCY concentrations (0.1–5 mM), such concentrations are similar to those used in many previous experimental studies in vitro [13–15]. Previous works showed that extracellular HCY concentrations in the millimolar range may be required to produce a significant rise of intracellular HCY [16], which may explain the need for relatively high concentrations of HCY in the experimental setting. Notwithstanding these limitations, our findings obtained at concentrations of 0.1–0.5 mM HCY are clinically relevant, as such concentrations can be reached in intermediate (30–100 μM) and severe (>100 μM) forms of hHCY in humans.

HCY-induced necrosis, as observed in the present study, has been reported only in a few experimental works, performed in neurons [17] and vascular smooth cells [18], but the underlying mechanisms remained elusive. In contrast, apoptosis has been frequently reported in endothelial cells, contributing to the proatherogenic effects of HCY [1]. Importantly, the activation of caspase-3 has been shown to be essential in the process of HCY-induced apoptotic cell death [1], by cleaving several nuclear proteins, including PARP and resulting in the internucleosomal cleavage of DNA and cell death [19]. Our findings of progressively increased cleavage of caspase-3 and PARP, together with DNA fragmentation in response to HCY (Fig. 2), are thus entirely consistent with these previous findings in endothelial cells.

A key mechanism of HCY-dependent endothelial toxicity is oxidative stress, due mainly to the upregulation of the superoxide ($O_2^{\cdot -}$)-producing enzyme NADPH oxidase [2,4,20]. A major reaction of $O_2^{\cdot -}$ is that with NO to form the potent oxidant peroxynitrite [11]. Accordingly, reduced NO availability and generation of peroxynitrite have been demonstrated in endothelial cells exposed to HCY [19–21]. Here, we provide the first demonstration that HCY also triggers peroxynitrite formation in a cardiac cell line, evidenced by a markedly increased intracellular generation of 3-NT (Fig. 3). Such formation of peroxynitrite largely explains the cytotoxicity of HCY, in view of the significant protection afforded by the peroxynitrite decomposition catalyst FeTPPS, which abolished 3-NT formation and significantly reduced the release of LDH, the fragmentation of DNA, the cleavage of caspase-3 and PARP, and restored normal cell morphology after HCY treatment (Fig. 4). Consistent with the effects of FeTPPS, we found

that the NOS inhibitor L-NAME also significantly reduced LDH release after HCY (Fig. 4E). Overall, these data strongly support the concept that HCY induces NO formation and peroxynitrite generation to kill H9C2 cardiomyocytes in vitro. Importantly, we recently showed that peroxynitrite endogenously produced during myocardial infarction in vivo induces cardiomyocyte apoptosis, through a pathway involving caspase-3 activation and PARP-1 cleavage [10]. These different sets of data suggest that peroxynitrite formation might represent a “final common pathway” linking different kinds of stresses to the induction of programmed cell death in the heart.

Another important finding of our study was the ability of HCY to activate the MAP kinases ERK and JNK in primary ventricular myocytes (Fig. 3C). This finding is consistent with the capacity of HCY to generate peroxynitrite, since we previously reported that peroxynitrite acts as a potent activator of JNK and ERK in cardiomyocytes [12]. This finding is particularly noticeable, since ERK and JNK are major stress signaling pathways linked with apoptotic cell death and myocardial hypertrophy [22,23]. Our observations may thus provide a plausible mechanism explaining why patients with hHCY present an increased risk for adverse cardiac remodeling, myocardial hypertrophy, and chronic heart failure [24].

In conclusion, the present results indicate that HCY elicits necrotic and apoptotic cell death in H9C2 cardiomyocytes through the intracellular generation of peroxynitrite, and also activates two major stress kinases (ERK and JNK) in primary cardiomyocytes. Thus, homocysteine appears to be directly detrimental to the myocardium, independently from its known deleterious effects on blood vessels.

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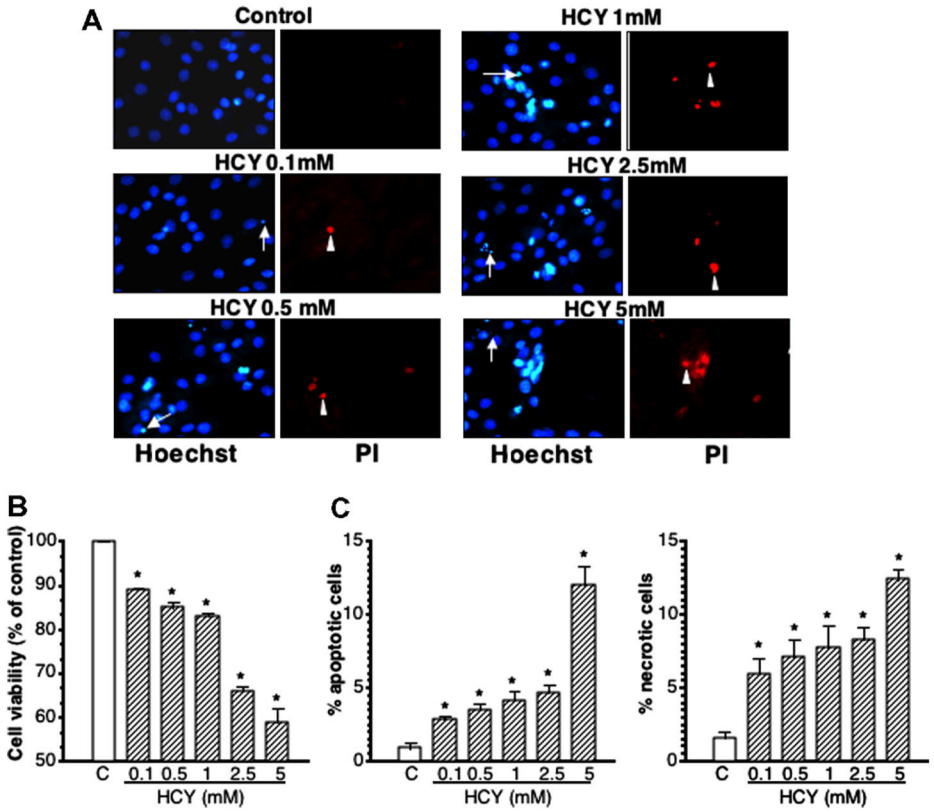

Fig. 1.

Homocysteine induces both necrotic and apoptotic cell death in H9C2 cardiomyocytes. (A) H9C2 cells treated for 6 h with 0.1–5 mM homocysteine (HCY) disclosed signs of both apoptosis (chromatin condensation and nuclear shrinkage, Hoechst staining, arrows) and necrosis (normal-sized nuclei stained with PI, arrowheads). (B) HCY at all concentrations reduced cell viability, assessed by MTT. (C) Apoptosis and necrosis were quantified in 8 random fields of at least 4 pictures per experimental condition. Magnification 40×. Graphs are means ± sem of at least 3 independent experiments. *$p < 0.05$ vs control.

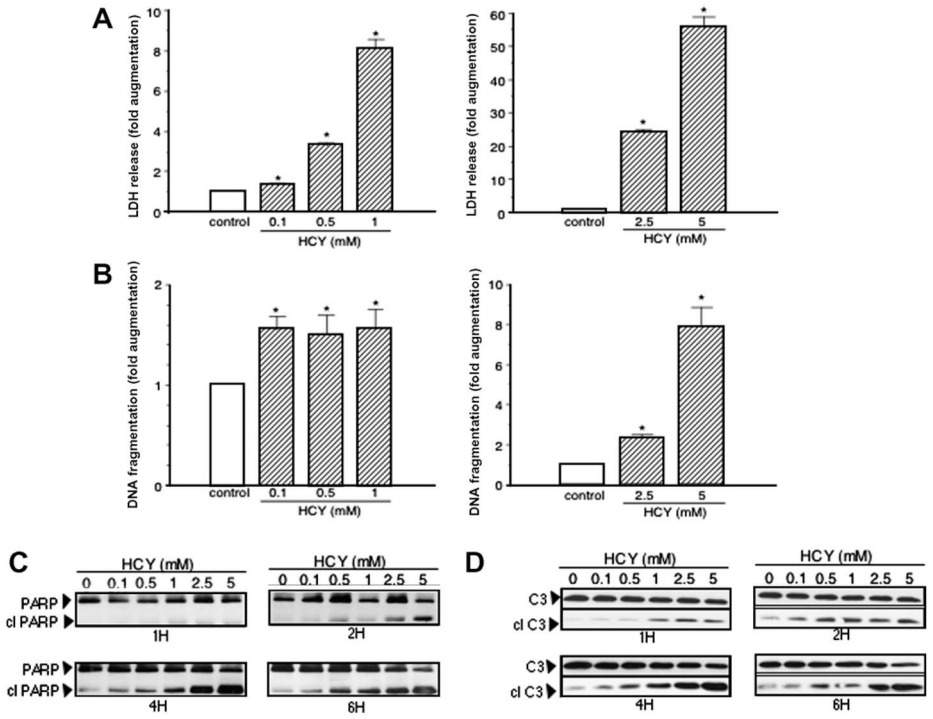

Fig. 2.

Homocysteine induces LDH release, DNA fragmentation and cleavage of PARP and caspase-3 in H9C2 cells. H9C2 cells treated with HCY (0.1–1 mM, left panel; 2.5–5 mM, right panel) for 6 h released LDH (A) in the medium (an index of cell necrosis), and showed oligonucleosomal DNA fragmentation (B), indicative of apoptosis. HCY also triggered the cleavage of both PARP (C) and caspase-3 (D), in a time- and concentration-dependent manner. Means ± sem of at least 3 independent experiments. *$p < 0.05$ vs control.

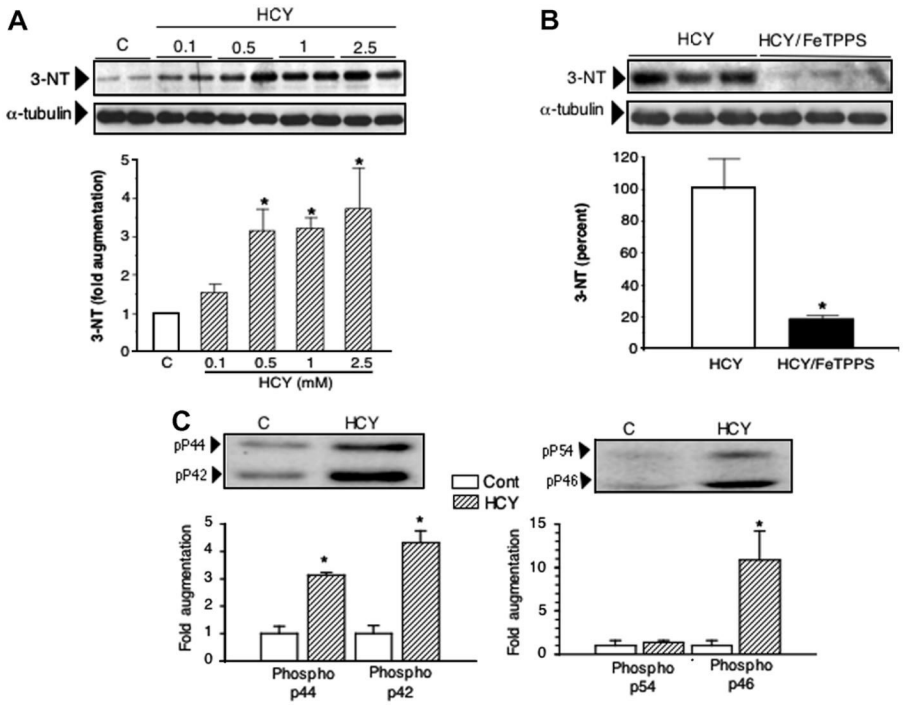

Fig. 3.

Homocysteine induces the intracellular generation of peroxynitrite in H9C2 cells, and activates ERK and JNK MAP kinases in primary rat ventricular myocytes. (A) H9C2 cells exposed to HCY at 0.1–2.5 mM for 6 h disclosed an increase in 3-nitrotyrosine (3-NT) formation, indicative of peroxynitrite generation, which was statistically significant at 0.5 mM HCY and above. (B) The formation of 3-NT elicited by HCY was completely abrogated by FeTPPS pretreatment. The levels of α-tubulin are shown as loading controls. (C, insert). Primary rat ventricular myocytes treated with HCY (1 mM, 6 h) showed an increased phosphorylation of ERK 1/2 (p44/p42) and JNK 1 (p46), whereas the phosphorylation of JNK2 (p54) was only modest and did not reach statistical significance. There was no change in the levels of nonphosphorylated ERK and JNK proteins (not shown). Graphs are means ± sem of at least 3 independent experiments. *$p < 0.05$ vs control.

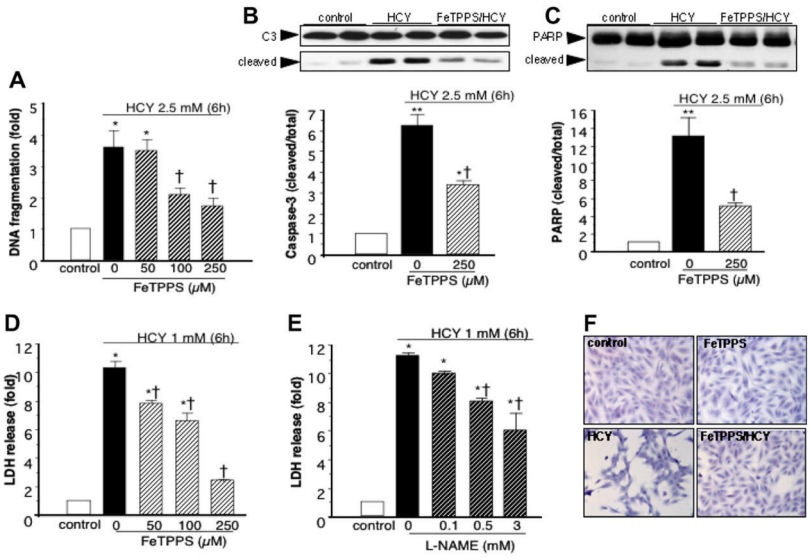

Fig. 4.

The peroxynitrite decomposition catalyst FeTPPS alleviates apoptotic and necrotic cell death triggered by HCY in H9C2 cells. (A) DNA fragmentation triggered by HCY (2.5 mM, 6 h) was significantly prevented by pretreatment (2 h) with FeTPPS at 100 and 250 μM. Accordingly, FeTPPS also significantly reduced the cleavage of PARP (B) and almost suppressed the cleavage of caspase-3 (C) triggered by HCY (2.5 mM, 6 h). (D) LDH release induced by HCY (1 mM, 6 h) was significantly prevented by pretreatment with FeTPPS in a concentration-dependent manner. (E) Cell necrosis, evaluated by LDH release, was also significantly prevented by pretreatment with the NO synthase inhibitor L-NAME at 0.5 and 3 mM. (F) The morphology of H9C2 cells was markedly altered after treatment with HCY (1 mM, 6 h). Whereas FeTPPS alone did not affect cell morphology, it restored normal cell morphology in conditions of HCY treatment. Magnification 40×. Graphs are means ± sem of at least 3 independent experiments. *$p < 0.05$ vs control; †$p < 0.05$ FeTPPS vs HCY alone.