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Effects of angiotensin II on vascular endothelial cells: formation of receptor-mediated reactive nitrogen species

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Abstract

Angiotensin II (ANG II) participates in many cardiovascular disease states, but the mechanisms involved are not completely defined. Doses of ANG II that do not affect blood pressure significantly can still cause early changes in vascular endothelial performance and cell-specific protein 3-nitrotyrosine formation (protein-3NT, marker of peroxynitrite formation) *in vivo*. Here, we have tested the hypothesis that ANG II induces endothelial cell peroxynitrite ($ONOO^-$) formation *in vitro*, and investigated the mechanisms involved. Endothelial cells were incubated with ANG II (1 nM–250 μM), and protein nitration was assessed by immunoblotting. ANG II caused concentration-dependent increases in protein-3NT above detectable basal control levels, at concentrations greater than 100 nM. This response was inhibited significantly by co-incubation with losartan or diphenyleneiodonium chloride. Endothelial cell lysates incubated with nitrated protein standards demonstrated significant protein-3NT modification activity only in the presence of serum. However, endothelial cell lysates did not modify the free amino acid form of 3NT (free-3NT) in identical experimental conditions, assessed by capillary electrophoresis. Finally, free-3NT was cytotoxic to cultured endothelial cells (fitted $LC_{50} = 98$ μM). These data demonstrate that stimulation of angiotensin receptor subtype 1 by ANG II can cause increased endothelial cell protein nitration *in vitro* in the absence of other cell types or stimuli, at concentrations that are pathophysiologically relevant. Furthermore, endothelial cells selectively modified nitrated protein tyrosine residues only in the presence of a cofactor(s), and did not modify the free modified amino acid. Protein nitration may be a regulated endothelial signaling process, while free-3NT may be toxic to endothelial cells.

Keywords: Nitric oxide; Endothelium; Reactive nitrogen species; Angiotensin II; 3-Nitrotyrosine; Peroxynitrite

1. Introduction

ANG II is an important participant in a broad spectrum of cardiovascular disease states, and pharmacological agents aimed at modulating its production (angiotensin converting enzyme inhibitors) and/or activities (ANG II receptor antagonists) have become mainstays of cardiovascular medicine [1–3]. Interestingly, the cardiovascular benefits of these drugs may be only partially related to their blood pressure-lowering effects, since in addition to its vasopressor effects, ANG II can directly modulate vascular cell growth, differentiation, and gene expression pathways [4,5]. More recent studies have demonstrated that ANG II promotes the formation of reactive oxygen species (particularly superoxide anion), and that this may also be an important component of ANG II-mediated cardiovascular disease [6]. Additionally, the putative sites of action of ANG II in cardiovascular disease may be more complex than originally thought: ANG II signaling occurs in multiple vascular cell types (smooth muscle cells, fibroblasts,

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Abbreviations: ANG II, angiotensin II; DPI, diphenyleneiodonium chloride; EYTA, 5,8,11,14-eicosatetraynoic acid; 3NT, 3-nitro-L-tyrosine; Protein-3NT, protein-bound 3-nitrotyrosine; Free-3NT, free amino acid form of 3-nitrotyrosine; NO, nitric oxide; $ONOO^-$, peroxynitrite; TNM, tetranitromethane.

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vascular endothelial cells), and clinical reports (Trial on Reversing Endothelial Dysfunction, TREND) have suggested that the benefits of ANG II inhibition may extend beyond cardiac and vascular smooth muscles (e.g. treatment with ACE inhibitors improved endothelial function in patients with coronary artery disease) [7–11].

The vascular endothelium plays a critical role in the regulation of local hemodynamics and vascular cell adhesion by the production and release of multiple humoral factors [12–14]. One critical mediator of endothelial function is NO, which under normal physiological conditions provides local anti-thrombotic actions and regulation of vasomotor tone [15]. The actions of NO can be altered severely under conditions of oxidative stress [16]. Of particular importance is the avid interaction of NO with superoxide anion ($O_2^{\bullet -}$), which results in the formation of the highly aggressive reactive nitrogen species $ONOO^-$ [17,18]. Relative to other biological oxidants, $ONOO^-$ possesses a high affinity to nitrate tyrosine residues, both protein-bound and free, forming 3NT [19]. Therefore, in addition to the loss of important NO-mediated signaling events, oxidative destruction of NO can have direct cytotoxic actions as well. Elevations in both protein- and free-3NT (plasma, cerebrospinal fluid) are relevant phenomena in human cardiovascular disease [20–22]. However, the critical stimuli inducing these events, the putative cellular targets affected, and the cellular defense mechanisms to accommodate these changes remain incompletely defined.

Although ANG II is a well-described effector in vascular smooth muscle, the mechanisms by which ANG II modulates endothelial function remain poorly understood. We have demonstrated recently that acute subpressor doses of ANG II cause selective impairment of vascular endothelial performance and protein nitration, in the absence of known vascular smooth muscle effects (altered contractile responses or morphological alterations) [23]. These findings suggested that ANG II may cause important alterations in vascular endothelium prior to actions on vascular smooth muscle *in vivo*, and that endothelial protein nitration may participate in this phenomenon. Here, we tested the hypothesis that ANG II can cause direct endothelial protein nitration *in vitro* in the absence of other cell types and/or physiological stimuli; we also defined mechanistic aspects of this interaction through the use of selective inhibitors.

Another important aspect of our studies was to assess the capacity of endothelial cells to modify nitrated tyrosine residues. Several recent reports have shown that various homogenized tissues possess some capability to metabolize nitrated proteins [24,25], but no previous studies have assessed endothelial cell metabolic capabilities. Since we have also shown recently that free-3NT is directly toxic to vascular endothelial cells [26], we tested the ability of endothelial cells to modify free-3NT versus protein-bound forms.

2. Materials and methods

2.1. Isolation and culture of murine endothelial cells

Endothelial cells were isolated from male C57BL mice, as previously described [27] and maintained in modified murine endothelial cell medium containing (per 500 mL): Dulbecco's modified Eagle's medium, 200 mL; Hams F12, 200 mL; Penn/Strep mixture, 8 U/mL of penicillin G sodium + 800 μg/mL of streptomycin sulfate; endothelial cell growth medium, 15 mg; heparin 5000 U (Gibco-BRL).

2.2. ANG II cell incubations

Cultured endothelial cells were seeded onto 96-well plates at 3×10^4 cells/mL. After establishing 90% confluence, fresh medium + 20% fetal bovine serum containing 0–250 μM ANG II (Calbiochem-Novabiochem Corp.) was added to each well. ANG II degradation effects were addressed by replacing the medium every 12 hr thereafter. After 24 hr of incubation, medium was removed, and cells were washed with PBS and then harvested using lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris, pH 7.4) for immunoblot analysis.

In a parallel set of experiments, cells were incubated with ANG II (100 μM) and one of the following compounds: losartan [angiotensin receptor subtype 1 antagonist] (Merck & Co.); DPI [flavoprotein (NADH/NADPH oxidase) antagonist], oxypurinol [xanthine oxidase antagonist], *N*-acetylcysteine [thiol-based antioxidant], EYTA [lipoxygenase antagonist], or aminoguanidine [nitric oxide synthase 2 antagonist] (Sigma Chemical Co.) at concentrations ranging from 250 nM to 500 μM. Medium supplemented with ANG II + inhibitors was replaced every 12 hr and then harvested for protein after 24 hr, identical to procedures described above.

2.3. Dot blot analyses for endothelial cell protein nitration

Immunoblot analyses for protein-3NT were performed, as previously described [20]. Two micrograms of protein from each cell lysate sample was adjusted to 100 μL with PBS and then blotted onto nitrocellulose membranes (0.2 μm pore size, Schleicher & Schuell) with the aid of a 96-well Bio-Dot Microfiltration Apparatus (Bio-Rad). Total protein was visualized using a reversible protein stain (BLOT-Fast Stain, Geno Technology Inc.). Then membranes were immunostained for tyrosine nitration using a polyclonal primary antibody raised against 3NT (anti-3-NT, Upstate Biotechnology; 1:400), as we have described previously [20]. Bands were visualized by enhanced chemiluminescence. Preincubation of primary antibody with free-3NT (5 mM) completely quenched positive staining, whereas tyrosine (5 mM) had no effect. Non-immune serum (isotypic) controls also showed no

detectable immunoreactivity in any treatment group, demonstrating antibody specificity.

Digital images of dot blot results (from both total protein and 3NT immunoreactivity stains) were captured, and then analyzed using research-based digital image analysis software (Image Pro Plus, Media Cybernetics). Images were background corrected, and average pixel intensities were determined. Untreated and TNM-treated BSA were used to develop standard curves of enhanced chemiluminescence signal versus moles 3NT by adding these to the cellular lysates and assuming 10% conversion of available BSA tyrosine residues [24]. In all cases, the relationship of protein-3NT immunoreactivity was linearly related to the amount of nitrated BSA added to the cellular lysates (e.g. the standard curve was linear within the range investigated, 1–200 pmol protein-3NT per well). Comparison of triplicate standard curves developed with TNM-treated BSA (positive control for protein-3NT) yielded a detection limit (twice background signal) of approximately 0.1 pmol 3NT. Coefficients of variation for this method were less than 5% for intra-blot variability, and equal to 16% for inter-blot variability.

2.4. Denitration studies

After washing with PBS, confluent cells were harvested from 75-cm^2 culture flasks in PBS buffer (Invitrogen/Gibco) containing protease inhibitors (10 μg/mL each of soybean trypsin inhibitor, aprotinin, leupeptin, prostatin A; 200 μM phenylmethylsulfonyl fluoride; 100 μM EDTA). Then cells were disrupted using an ultrasonic probe at 4° (Fisher Scientific). The resulting cell lysate (endothelial cell protein + PBS buffer + protease inhibitors) was then studied for its capacity to modify protein nitration chemistries by supplementing the cell lysate with nitrated substrate (nitrated BSA), and measuring the reduction in protein-3NT signal following increasing incubation times. Three milliliters of cell lysate (total protein concentration 2 mg/mL) was incubated with 0.5 mL of nitrated BSA (1 mg/mL) at 37° with or without supplementation with 20% fetal bovine serum. Serum alone controls showed no activity. Samples from this incubation mixture (500 μL each) of cell lysate and nitrated BSA were collected at different time points (1, 6, 18, and 24 hr) and boiled for 10 min in the presence of 2% SDS to stop conversion. Protein concentration in each sample was then determined by a bicinchoninic acid protein assay, and evaluated for protein nitration using the immunoblot assay as described above.

In parallel studies, we examined the capacity of endothelial cells to modify free-3NT; endothelial cell lysates were incubated with free-3NT (with and without 20% fetal bovine serum) for 36 hr, and assessed for free-3NT content at 0, 1, 6, 18, and 24 hr (see capillary electrophoresis assay below). A serum (cell-free) control was performed by incubation of 20% serum without cell lysate. A decrease in protein or free-3NT signal following incubation with the lysates would indicate a modification of the nitro group in the nitrated tyrosine, and may indicate a specific capacity of endothelial cells to modify protein versus free-3NT.

2.5. Capillary electrophoresis assay for free-3NT

Free-zone capillary electrophoresis was conducted with a P/ACE 5510 system (Beckman Instruments Inc.) in an uncoated fused-silica capillary. Electrophoretic separation was performed in 50 mM sodium borate/50 mM SDS buffer, pH 9.3, at 20 kV, 25°, using UV detection at 200 nm. Standard curves for the simultaneous separation of free-3NT, Tyr, and 3-amino-L-tyrosine (reduced end-product of 3NT) were generated using endothelial cell lysates spiked with free-3NT, Tyr, or 3-amino-L-tyrosine, respectively. Migration times for 3-aminotyrosine, Tyr, and free-3NT were 4.939 ± 0.029 min (coefficient of variation = 0.6%, N = 45), 5.3 ± 0.04 min (coefficient of variation = 0.8%, N = 45), and 6.8 ± 0.07 min (coefficient of variation = 1.1%, N = 45), respectively. Intra- and inter-day assay variabilities were 1.7 and 2.3%, respectively. The mass limit of detection for free-3NT in the endothelial cell lysate was approximately 100 amol on capillary. Preliminary studies included treatment of free-3NT-spiked endothelial cell lysates with 1 mM sodium hydrosulfite as a positive control for the modification of free-3NT to free aminotyrosine. Over 90% of free-3NT was converted at 1 hr post-treatment; stoichiometric conversion of free-3NT was achieved following treatment for 24 hr.

2.6. Cell viability assay

Endothelial cells (4×10^4) were seeded in UV transparent 96-well plates, grown to confluence for 24 hr, and then treated for 24 hr in medium alone or in medium fortified with 1 nM–250 μM ANG II, 1 μM–1 mM free Tyr, 1 μM–1 mM free-3NT, 50 nM–50 μM BSA, or 500 nM–500 μM TNM-treated BSA (protein concentrations chosen to produce equimolar cellular exposures of protein-bound Tyr or 3NT). Next, cells were washed and fixed in 5% buffered formalin, and stained with crystal violet as a marker of cell viability, as previously described [28]. The crystal violet signal was assayed spectrophotometrically at 590 nm. Preliminary experiments involved the production of a standard curve of crystal violet intensity to various dilutions of endothelial cells; intra- and inter-day variabilities were each less than 5%.

2.7. Data analysis and statistics

Data are presented as means ± SEM. Where appropriate, cell viability data were fit to the four parameter logistic equation, using GraphPad Prizm software. Statistical analyses were performed using one-way analyses of variance, with *post hoc* Student–Newman–Keuls tests to

evaluate significant differences. Statistical significance was assigned at $P < 0.05$.

3. Results

3.1. ANG II-induced protein-3NT formation in isolated endothelial cells

Preliminary studies verified normal endothelial cell growth in culture medium. Initial seeding of 1×10^4 cells/mL of medium resulted in cell population doubling on day 3, and cells reached 90% confluence on day 5 (data not shown). In these studies we focused on studying endothelial cells at confluence and, therefore, only employed cells after 5 days of culture.

The effect of ANG II incubation on the extent of endothelial cell protein nitration is illustrated in Fig. 1. Protein nitration was evaluated by immunoblot analysis at 24 hr post-treatment. Control incubations of endothelial cells in the absence of ANG II yielded detectable basal levels of protein-3NT. ANG II treatment caused concentration-dependent increases in endothelial cell protein nitration, at concentrations as low as 100 nM, within ranges that are likely physiologically and/or pathophysiologically relevant ($P < 0.05$ versus control at 0.1 to 250 μM ANG II). Incubations for 24 hr with ANG II were not cytotoxic to cultured endothelial cells at any of the concentrations studied, as determined by the crystal violet assay (data not shown).

In parallel experiments, potential enzymatic sources of increased NO and/or $O_2^{\bullet -}$ were probed for activity in this setting by the use of selective inhibitors (Fig. 2). Co-incubation with losartan (angiotensin receptor subtype 1 antagonist), DPI (NADH/NADPH oxidase antagonist), and *N*-acetylcysteine (thiol antioxidant) all significantly inhibited protein nitration in endothelial cells ($P < 0.05$ versus 100 μM ANG II alone). The order of potency in blocking protein-3NT formation was losartan ($IC_{50} \sim 10$ nM) > DPI ($IC_{50} = 415 \pm 8.6$ nM) > *N*-acetylcysteine ($IC_{50} = 2.04 \pm 0.19$ μM). EYTA, oxypurinol, and aminoguanidine had no detectable inhibitory activity on endothelial cell protein nitration events.

3.2. Endothelial cell-mediated modification of protein-3NT versus free-3NT

Using nitrated BSA as a substrate, the capacity of endothelial cells to modify protein nitration events in the presence and absence of serum was investigated. Shown in Fig. 3 are the relative time-courses for endothelial cells to modify nitrated BSA in the presence and

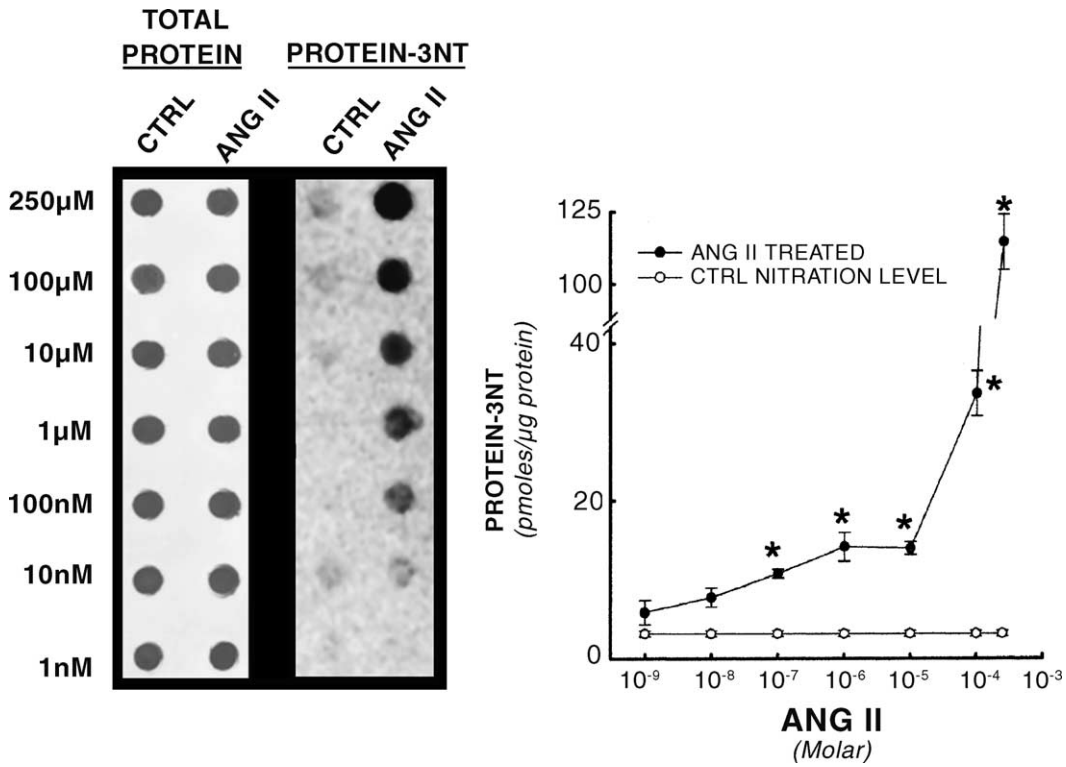

Fig. 1. ANG II-induced endothelial cell protein-3NT formation. Cells were incubated with ANG II (1 nM–250 μM) or vehicle control (medium + serum alone) for 24 hr. Total protein and molar protein-3NT formation were detected by dot blot as described in Section 2. Left panel: representative dot blot images for total protein and protein-3NT. Total protein load (2 μg endothelial cell lysate) was equivalent in each well. Right panel: mean concentration–effect relationships for ANG II-treated endothelial cells. ANG II elicited a statistically significant increase in protein-3NT formation at concentrations greater than 100 nM. Values are means ± SEM, N = triplicate experiments. Key: (*) $P < 0.05$ compared with control (0 μM ANG II).

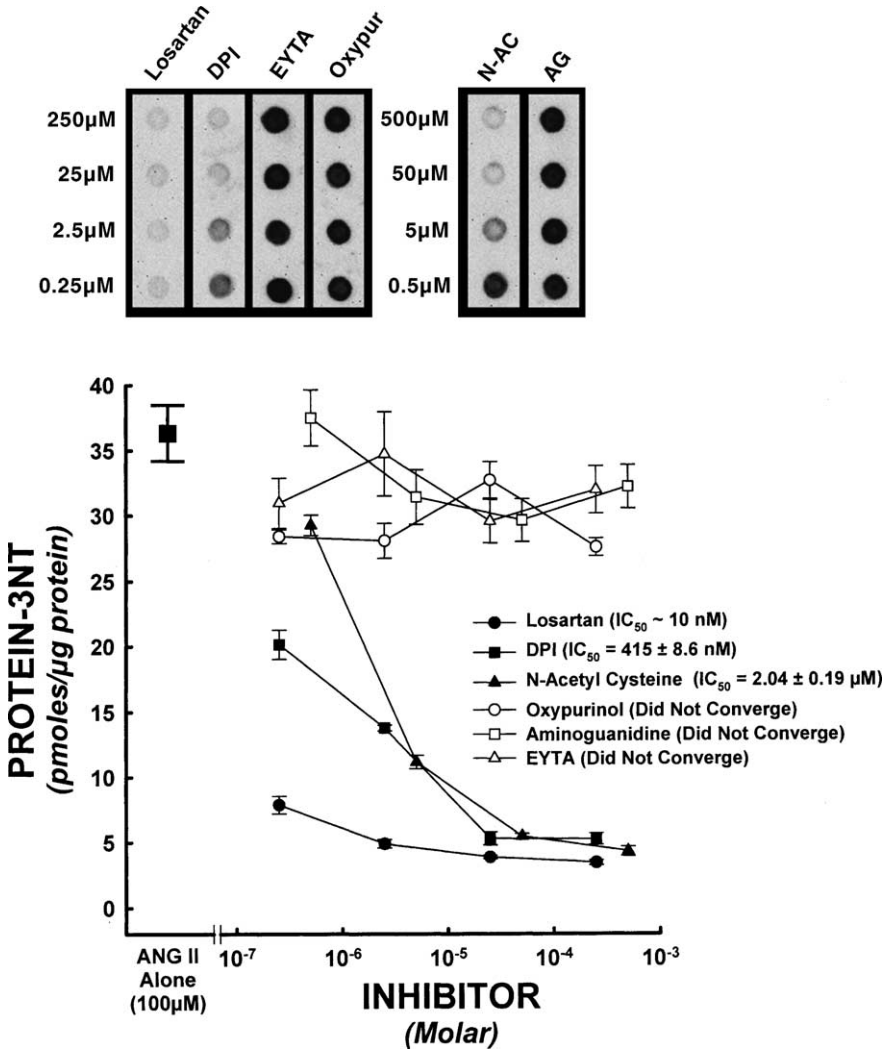

Fig. 2. Endothelial cell nitration mediated by activation of the angiotensin receptor subtype 1. Cells were incubated with ANG II (100 μM) + a selective inhibitor [losartan, angiotensin receptor subtype 1 antagonist; DPI, flavoprotein (NADH/NADPH oxidase) antagonist; EYTA, lipoxygenase antagonist; oxypurinol (Oxypur), xanthine oxidase antagonist; *N*-acetylcysteine (*N*-AC), thiol-based antioxidant; or aminoguanidine (AG), nitric oxide synthase 2 antagonist; concentrations 250 nM–500 μM] for 24 hr. Total protein and molar protein-3NT formation were detected by dot blot as described in Section 2. Upper panel: representative dot blot images for endothelial cell lysates incubated with ANG II + inhibitors probed for protein-3NT. The total protein load (2 μg endothelial cell lysate) was equivalent in each well. Lower panel: mean concentration–effect relationships for inhibitors. Losartan, DPI, and *N*-AC had a statistically significant inhibitory effect on ANG II-mediated increases in protein-3NT formation ($P < 0.05$ compared to control). Values are means ± SEM, N = triplicate experiments.

absence of serum supplementation. Cell lysates were incubated with nitrated BSA (150 μg/mL) in the presence and absence of fetal bovine serum (20% by volume). All cell lysates contained protease inhibitors (see Section 2). No degradation of the protein-3NT signal was observed at 24 hr in cell-free controls. Significant nitration modifying ability was observed at 24 hr ($P < 0.05$) in cell lysates containing fetal bovine serum, compared to cell-free or serum alone controls (Fig. 3). An apparent zero-order rate was observed with approximately 50% of protein-3NT converted in 12 hr. In contrast, no significant modification of protein-3NT was seen in the absence of serum or with serum alone, as serum-free endothelial cell protein-3NT content was not significantly different from cell-free controls.

Using the free modified amino acid, free-3NT, as a substrate, the capacity of endothelial cells to modify free nitrated tyrosine was determined in parallel studies, using capillary electrophoresis and UV detection to quantify free-3NT levels in endothelial cell lysates. Shown in Fig. 4 are representative electrophoreograms for free-3NT in endothelial cell lysates, before and after treatment with 1 mM sodium hydrosulfite (conducts chemical reduction of nitrotyrosine to aminotyrosine). In the upper profile, a representative, baseline resolved peak for free-3NT is shown; following 24 hr of hydrosulfite incubation, the free-3NT peak was ablated, and formation of 3-aminotyrosine was observed. Endothelial cell lysates were analyzed for the capacity of free-3NT (200 μM) to alter nitration in the presence and absence of serum, as described above.

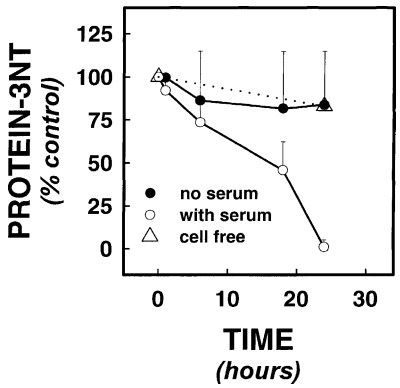

Fig. 3. Modification of nitrated BSA by endothelial cell lysates. Decreases in 3NT immunoprevalence in endothelial cell lysates incubated with nitrated BSA (no fetal bovine serum versus 20% fetal bovine serum) were detected by dot blot immunoreactivity analysis at 1, 6, 18, and 24 hr. The absolute value for protein-3NT in 500 μg nitrated BSA (at time 0) = 151.5 nmol (a starting concentration of approximately 40 μM in protein-bound nitrated tyrosine residues). The serum control was performed by incubation of 20% serum and nitrated BSA without the cell lysate. Values are means ± SEM, N = triplicate experiments.

In contrast to protein-3NT studies, no significant modifying capacity was observed in endothelial cell lysates against the free modified amino acid (Fig. 4).

3.3. Endothelial cytotoxicity of protein-3NT versus free-3NT

The relative cytotoxicities of exogenous addition of free versus protein-3NT were determined using a crystal violet assay for endothelial cell viability (Fig. 5). Free-Tyr or free-3NT (1–1000 μM) was applied to cultured endothelial cells for 24 hr, and cell viability was assessed. Free-3NT caused concentration-dependent decreases in cell number after 24 hr, with a fitted LC_{50} of 98 ± 7.5 μM. Equimolar Tyr had no effect. Concentrations of BSA (50 nM–50 μM) and nitrated BSA (500 nM–500 μM) were chosen to achieve an equivalent exposure of Tyr or 3NT in the protein-bound form, based on the relative number of Tyr residues and estimated nitration efficiency (see Section 2). Neither protein treatment had any significant effect on cell density at 24 hr.

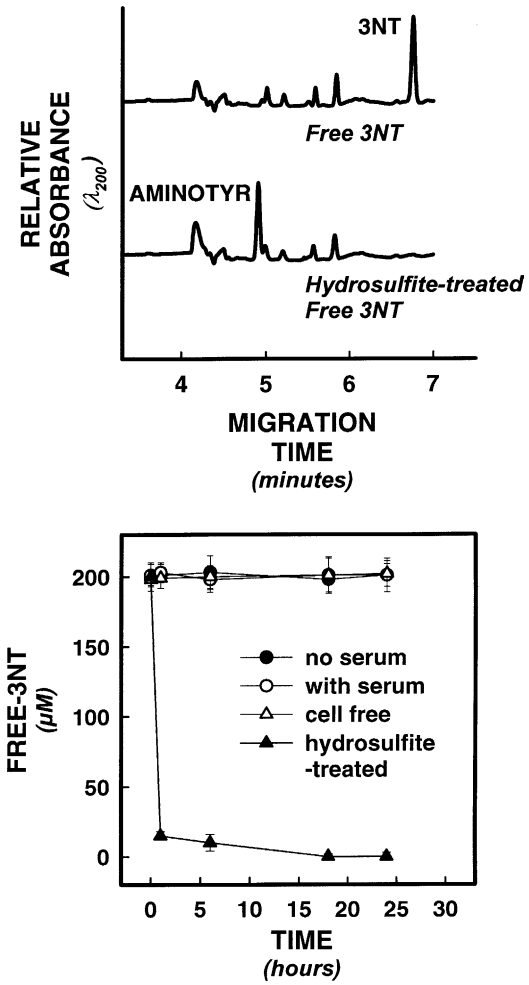

Fig. 4. Failure of endothelial cell lysates to modify free-3NT. Endothelial cell lysates were incubated with free-3NT (no fetal bovine serum versus 20% fetal bovine serum) for 36 hr, and assessed for 3NT content at 0, 1, 6, 18, and 24 hr. A serum (cell-free) control was performed by incubation of 20% serum and free BSA without the cell lysate. A free-zone capillary electrophoresis method was developed to detect free-3NT and its chemically reduced end-product, 3-aminotyrosine. Upper panel: representative electrophoreogram of the separation of 3NT and 3-aminotyrosine in endothelial cell lysates before and after chemical conversion by 1 mM sodium hydrosulfite. Lower panel: endothelial cell lysates were assayed for free-3NT content over 24 hr. No significant modification of free-3NT content was observed in any treatment group. Values are means ± SEM, N = triplicate experiments.

4. Discussion

Increased oxidative stress has been implicated in the initiation and/or progression of a variety of acute and progressive vascular disease states, including ischemia–reperfusion injury, hyperlipidemia, atherosclerosis, and essential hypertension [29,30]. Studies by us and other investigators have demonstrated that formation of reactive nitrogen species (including peroxynitrite and others) and attendant tyrosine nitration may be particularly injurious events in the development of cardiac and vascular dysfunction [14,16,18–23,26,31]. However, the mechanisms by which nitration occurs, and the factors that modulate cellular vulnerability to reactive nitrogen species formation are not understood completely. Although ANG II is known to induce superoxide anion formation, most mechanistic studies have focused on its effects on vascular smooth muscle, not its potential endothelial toxicities via interactions with NO. We recently demonstrated that ANG II-induced endothelial nitration and dysfunction precede other vascular alterations *in vivo* [23]. While nitration events were implicated in our previous *in vivo* experiments, the cell type(s) responsible and the endothelial response to injury have not been investigated. Here, we tested the hypothesis that an ANG II challenge causes increased

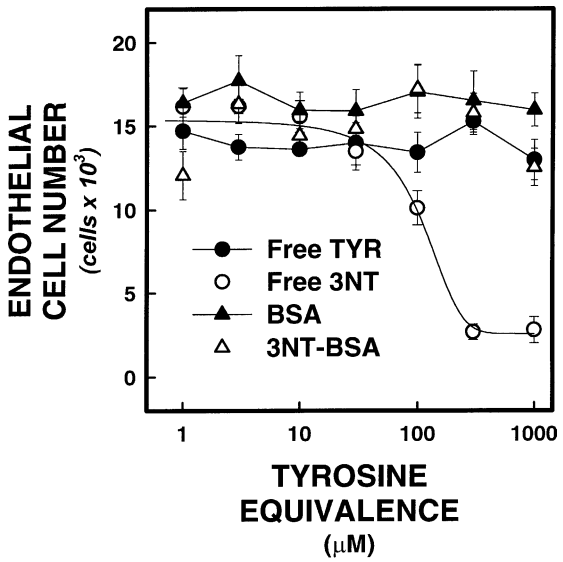

Fig. 5. Selective endothelial cytotoxicity of free-3NT. Endothelial cells were treated for 24 hr in medium alone or medium fortified with 1 μM–1 mM free-Tyr, 1 μM–1 mM free-3NT, 50 nM–50 μM BSA, or 500 nM–500 μM TNM-treated BSA (3NT-BSA), protein concentrations chosen to produce equimolar cellular exposures of protein-bound Tyr or 3NT. Cells were stained with crystal violet as a marker of cell viability and assayed spectrophotometrically at 590 nm. Free-3NT elicited marked decreases in endothelial cell density; equimolar Tyr and protein-3NT had no effect. Fitted LC_{50} = 98 μM. Values are means ± SEM, N = triplicate experiments.

tyrosine nitration in isolated endothelial cells, independent of other cell types and/or physiological stimuli.

Consistent with our previous *in vivo* results, we found that ANG II caused significant increases in endothelial protein-3NT formation, at concentrations (submicromolar) likely to be pathophysiologically relevant [1,4–6]. These findings suggest that endothelial cells themselves have sufficient capacity to form reactive nitrogen species, in the absence of multi-cell interactions and/or complex physiological regulation that may occur *in vivo*. In subsequent experiments, we investigated potential sources of this protein-3NT formation, using selective inhibitors to the angiotensin receptor subtype 1 (losartan), NADH/NADPH oxidase (DPI), NOS2 (aminoguanidine), xanthine oxidase (oxypurinol), lipoxygenase (EYTA), or a thiol-based antioxidant (*N*-acetylcysteine). Inhibition experiments demonstrated that losartan, DPI, and *N*-acetylcysteine each had significant inhibitory activity on ANG II-mediated endothelial nitration. These effects support the hypothesis that ANG II-mediated protein-3NT formation resulted from AT1 receptor-mediated activation of the NADH/NADPH oxidase complex in these cells, resulting in increased superoxide anion formation [32]. Furthermore, these findings are consistent with recent studies regarding oxidative consequences of AT1 activation in other cell types as well [6,33,34]. Inhibitors of the inducible isoform of nitric oxide synthase, xanthine oxidase, and lipoxygenase were found to have no inhibitory effect (even at concentrations of 250–500 μM), suggesting that these pathways did not participate in ANG II-mediated protein-3NT formation in this setting.

Despite considerable evidence that $ONOO^-$ formation and attendant tyrosine nitration are mechanistic events in the development of cardiovascular disease, the cellular sites and responses to these events are largely unknown. While a variety of low molecular weight reductants (e.g. glutathione and ascorbic acid) may comprise an important cellular antioxidant defense system against $ONOO^-$ formation, no antioxidant defense system (i.e. superoxide dismutase or catalase) specific to $ONOO^-$ has been demonstrated in mammalian systems [18,19,31], placing particular emphasis on the recycling or metabolism of enzymatic targets modified by $ONOO^-$ as a potentially cytoprotective response to injury. Recent evidence has suggested that tissue homogenates (particularly those of the spleen, skin, and lungs) have the capacity to modify (or perhaps "denitrate") protein-3NT [24,35]. The cellular specificity of these events remains undefined, and the capacity of endothelial cells to conduct these chemistries has not been hypothesized. Here, we observed significant nitration-modifying capacities in isolated endothelial cells. This ability required certain undefined cofactors (activity was lost in the absence of serum), and had a half-time of 12 hr, less active than the tissue "denitrating" capacities of spleen and lung described above. These results constitute the first cell-specific evidence that modifying capacities are operable against nitrated proteins, and identify a potentially important endothelial cell detoxification pathway against the direct actions of $ONOO^-$. The mechanisms by which these modifications occur and the cytoprotective (or perhaps cytotoxic) potential of these events warrant further investigation.

Separate from increased protein-3NT in tissue samples, increased circulating levels of the modified free amino acid (free-3NT) have also been described in multiple settings of vascular endothelial dysfunction, and have been employed as a marker of $ONOO^-$ formation and/or increased nitrated protein catabolism in these settings [22,31,36]. We have shown recently that the free modified amino acid possesses intrinsic cytotoxic actions, inducing selective vascular endothelial dysfunction and endothelial cell apoptosis at clinically demonstrable concentrations [26]. Therefore, the capacity of endothelial cells to modify free-3NT may be particularly relevant to endothelial health and function. In studies parallel to those described for nitrated protein, we found that endothelial cells apparently do not modify free-3NT, in contrast to their capacity to modify protein-3NT. Further studies demonstrated that 24-hr exposures to free-3NT were cytotoxic to isolated endothelial cells, while equimolar Tyr and protein-3NT had no effect. The exogenous administration of these compounds only models extracellular endothelial exposures to 3NT (e.g. blood-borne or released by other cells), and may be most relevant to free-3NT studies. However, these findings further contribute to the shift in perspective of the free modified amino

acid from benign biological marker to potential mediator of oxidant-related damage. Since our assay detection limit was insufficient to detect free-3NT in ANG II-treated endothelial cells after lysis, the contribution of ANG II-mediated pathways to free-3NT formation in this setting remains undetermined. Other investigators have produced concurrent evidence that the biological reactivities of these two forms may be distinct [37–40]. Our evidence of differential modification capacities in the free versus protein-bound form may partially explain this phenomenon.

In summary, we found that ANG II stimulates isolated endothelial cell protein nitration in a concentration-dependent fashion, at concentrations as low as 100 nM. These findings are highly consistent with our *in vivo* results that ANG II stimulates endothelial cell nitration and functional impairment, prior to detectable effects in vascular smooth muscle function. Inhibition experiments demonstrated that this effect occurred via selective activation of NADH/NAPDH oxidase in an AT1 receptor-mediated process. In combination, these results suggest that the interactions of ANG II with endothelial cells may be a previously unrecognized and early event in the initiation and/or progression of cardiovascular dysfunction, and that strategies to selectively modulate the effects of ANG II on the endothelium may have therapeutic value. We also found that endothelial cells have the capacity to selectively modify protein-bound, but not free, nitrotyrosine, and that the free form of this modified amino acid was significantly more cytotoxic *in vitro*. Further studies regarding the potential contribution of ANG II-mediated endothelial cell injury to the initiation of cardiovascular disease, and the mechanisms of free-3NT endothelial cytotoxicity appear warranted.

Acknowledgments

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