Differential regulation of L-arginine transporters (cationic amino acid transporter-1 and -2) by peroxynitrite in rat mesangial cells

Idit Feenberg Schwartz, Tamara Chernichovsky, David Hagin, Meirav Ingbir, Ran Reshef, Gil Chernin, Yoram Levo and Doron Schwartz

Nephrology Department, Tel Aviv Sourasky Medical Center, Tel Aviv University, Sackler School of Medicine, Tel Aviv, Israel

Abstract

Background. It has become evident that increased nitric oxide (NO) generation may be associated with production of reactive oxygen species, such as peroxynitrite (ONOO\(^{-}\)). Peroxynitrite has been postulated to be responsible for several of the cytotoxic effects previously ascribed to NO. Since cellular arginine uptake has been shown to modulate nitric oxide synthase activity, we were intrigued to study the effect of ONOO\(^{-}\) on arginine traffic in renal mesangial cells.

Methods. Arginine uptake, CAT-1 and CAT-2 mRNA expression by northern blotting analysis, and CAT-1 protein content using western blotting were determined in mesangial cells pre-treated with peroxynitrite (0.1 and 0.5 mM) for 2 h.

Results. Peroxynitrite induced a significant increase in arginine uptake and CAT-2 mRNA expression compared with untreated cells. In contrast, CAT-1 mRNA expression and protein abundance were diminished.

Conclusions. In rat mesangial cells, peroxynitrite augments arginine uptake via augmentation of CAT-2 while decreasing CAT-1 expression.

Keywords: L-arginine transport; nitric oxide; reactive oxygen species

Introduction

Nitric oxide (NO) and oxygen-derived radicals such as superoxide radical (O\(_2^{-}\)), lipid peroxyl radicals (LOO\(^{\cdot}\)), hydroxyl radicals (OH\(^{-}\)) and peroxynitrite (ONOO\(^{-}\)) [4]. Peroxynitrite can easily penetrate cells in the protonated form because of its high diffusibility across phospholipid membranes [5]. It is known to initiate oxidative modification of proteins, including the nitration of aromatic rings, sulfoxidation of methionine and S-nitrosylation of cysteine followed by disulfide formation, thereby rendering inactive certain functionally important regulatory proteins like receptors or enzymes [6,7]. Peroxynitrite in particular has been postulated to be responsible for several of the cytotoxic effects previously ascribed to NO. For example, in iNOS-transfected human renal tubular epithelial cells, eliminating L-arginine from the medium was found to result in ONOO\(^{-}\) generation, nitrotyrosine formation and cellular injury [4].

Glomerular mesangial cells are directly exposed to substances circulating in the blood, which can easily pass through the fenestrated glomerular endothelium. Mesangial cells have been reported to express an inducible NO synthase in response to various cytokines with subsequent increased production of NO [8]. It is noteworthy that coincubation of mesangial cells with NO and (O\(_2^{-}\)) in a balanced ratio (which results in peroxynitrite generation) produces cross protection against the toxicity of each compound individually, suggesting that simultaneous generation of NO relative to (O\(_2^{-}\)) reflects a protective mechanism by antagonizing the destructive capacity of individually acting radicals [9,10].

Since tissue content of arginine has been suggested to affect local peroxynitrite generation and toxicity, we were intrigued to study the effect of ONOO\(^{-}\) on arginine traffic and to elucidate whether these changes are related to arginine transporters: cationic amino acid transporter-1 and -2 (CAT-1 and CAT-2), which represent the major carrier for cationic amino acids in most mammalian cells, referred to as the system y\(^{+}\) [11].
Materials and methods

All standard reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA unless indicated otherwise. $[^{3}H]$-arginine was supplied by Perkin Elmer, Life and Analytical Sciences, Boston, MA. Tetramethylammoniumperoxynitrite was purchased from Alexis Biochemicals, San Diego, CA.

Mesangial cell isolation and culture

Animal experiments described in this study were conducted in accord with the protocol approved by the institutional committee on ethics in animal experiments.

Wistar rats (3–4 weeks old) were sacrificed. Kidneys were surgically removed and kept on ice in sterile Dulbecco’s modified Eagle’s medium (DMEM) with 1% fetal calf serum (FCS). The kidneys were minced and glomeruli separated from the remaining renal tissue by sequential mechanical sieving as previously described [11]. Glomeruli were plated onto culture flasks using a selective Rosewell Park Memorial Institute (RPMI) medium (in which t-valine was substituted with l-valine to inhibit fibroblast growth) containing 15% FCS, streptomycin (10 mg/ml), penicillin (10 000 U/ml) and L-glutamine (2 mM) at 37°C. Media were replaced every 48 h, while adherent cells were retained. After cells reached confluence (in about 4 weeks), they were passaged using trypsin/ethylenediamine tetraacetic acid (EDTA). The cells utilized in these experiments exhibited typical morphological characteristics of mesangial cells and stained uniformly positive for $\alpha$-smooth muscle actin. Cells between passages 2 and 3 were used for subsequent experimental procedures. Experiments were performed 2 h following the administration of peroxynitrite (PN).

L-Arginine uptake in mesangial cells

Cells were seeded onto 6-well plates (Corning), at a density of $10^6$ cells per well. When confluent, cells were washed with 2 ml N-[2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid] (HEPES) buffer, pH 7.4. Arginine uptake was determined essentially as described by Gazolla et al. [12]. In brief, $[^{3}H]$-arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 1 ml for 1 min. The duration of 1 min was chosen since our preliminary studies have demonstrated that transport of 1 mM of $[^{3}H]$-arginine increased over time, and was linear at $\sim$1 min (data not shown). Transport activity was terminated by rapid aspiration of medium and washing the cells with ice-cold phosphate-buffered saline (PBS) (four times, 2 ml/plate). Cells were then dried and solubilized with 1 ml of 0.5% sodium dodecyl sulphate (SDS) in 0.5 N NaOH. To monitor radioactivity using liquid scintillation spectrometry (Betamatic, Kontron) 700 $\mu$l of the extract was used and the remaining 300 $\mu$l was used for protein content determination by Lowry method. To correct for non-specific uptake or cell membrane binding, cells were incubated with 10 mM unlabelled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. The results are expressed as mean $\pm$ SE of at least five different experiments.

Analysis of mRNA levels for CAT-1 and CAT-2 by northern blotting analysis

Total cellular RNA was extracted from cells following the method described by Chomczynski and Sacchi [13]. CAT-1 and CAT-2 mRNA levels were determined by northern hybridization. Of the total RNA, 15 $\mu$g were denatured and fractionated by size on 1.3% formaldehyde-agarose gel. RNA was transferred overnight, by capillary action, to a nylon membrane (Hybond-N, Amersham) and cross-linked by short-wave ultraviolet illumination. Purified end products of CAT-1, CAT-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (25 ng/ml) were used directly for radiolabelling following electrophoresis in 1.5% (w/v) low-melting point agarose gel. The probes used were labelled to a specific activity of $>10^9$ cpm/µg with $[^{32}P]$deoxycytidine 5’-triphosphate (dCTP) by a random primer labelling method (Gibco BRL). After hybridization with $^{32}$P-labelled cDNA overnight at 50°C, the membranes were sequentially washed twice in 1X saline sodium citrate (SSC), 0.1% SDS for 15 min, at room temperature, once in 1X SSC, 0.1% SDS for 15 min at 50°C, followed by 0.5X SSC, 0.1% SDS at 55°C for 15 min, and then washed at high stringency in 0.1X SSC, 0.1% SDS at 57°C for 15 min. Autoradiography was carried out with Kodak XAR film for 24-48 h at $-70^\circ$C. Relative mRNA abundance was quantified by measuring the density of the exposed film with a densitometer (B.I.S 202D). CAT-1 and CAT-2 mRNA levels were normalized to GAPDH mRNA, and were expressed in arbitrary units as the ratio of CAT-1 to GAPDH expression in three different experiments.

CAT-1 protein quantification by western blotting

CAT-1 protein expression in mesangial cells was determined by western blot analysis. Briefly, cells were placed in ice-cold PBS lysis buffer (pH 7.4), containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4.5 $\mu$M leupeptin and 5 $\mu$M aprotinin) (ICN Biomedicals Inc.), 0.01% Triton X-100 and 0.1% SDS, mechanically homogenized, and left on ice for 45 min. Homogenates were subsequently centrifuged (12 000 g rpm for 10 min at 4°C). Cell lysates were stored in aliquots at $-70^\circ$C. A membrane fraction was obtained by adding to the pellet an equal volume of lysis buffer supplemented by Tween-20 (0.25%) to solubilize. The protein content of each sample was determined by the method of Lowry. Equal amounts of protein (30 $\mu$g) were prepared in sample buffer (2% SDS, 0.01% bromophenol blue, 25% glycerol, 0.0625 M Tris–HCl, pH 6.8, 5% mercaptoethanol) and analysed on a 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham Corp.), and blocked in PBS-T containing 5% non-fat dried milk at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat CAT-1 antibodies: 1:500 and 1:200 for peptide-1 and peptide-2, respectively (synthesized by Dr O. Leitner, Weizmann Institute, Rehovot, Israel) for 1 h at room temperature, washed, and incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:10 000) in PBS-T for 1 h. Membranes were subsequently washed three times, for 5 min each, in PBS-T. Membranes were then stripped and reprobed with $\beta$-actin. CAT-1 was detected by short-wave ultraviolet illumination.
enhanced chemiluminescence (Kodak X-OMAT AR film) and quantified by densitometry.

Statistical analysis
Data are presented as the means ± SE. Data from the various experiments were subjected to ANOVA, and P-values < 0.05 were considered to be statistically significant.

Results
Initially, we aimed to determine the non-toxic doses of peroxynitrite by measuring lactate dehydrogenase (LDH) levels in the supernatant of cells exposed to different concentrations of the substance. Incubating mesangial cells with 1 mM of peroxynitrite for various lengths of stimulations (30 min, 1, 2 and 4 h) resulted in a ~4-fold increase in LDH levels compared with untreated cells. Using lower concentrations of peroxynitrite (0.1 and 0.5 mM) resulted in LDH release similar to controls and were therefore used throughout the study (data not shown). Exposing cells to the aforementioned concentrations of peroxynitrite for 0.5, 1, 2 and 4 h revealed that the most effective incubation period is 2 h (Figure 1).

The next set of experiments was performed to determine whether peroxynitrite affects arginine transport in mesangial cells. Incubation of mesangial cells with two different concentrations of peroxynitrite (0.1 and 0.5 mM) for 2 h resulted in doubling of arginine uptake velocities as compared with untreated cells (Figure 2). In order to confirm that the aforementioned phenomenon is related to peroxynitrite, cells were treated in the same way except that the ONOO⁻ solution was degraded previously in the buffer at 37°C for 15 min before being added to the cells. Arginine uptake in these cells was not different from untreated cells (410 ± 40 vs 430 ± 60 pmol arginine/mg protein/min, n = 4). Following the initial experiments, we wished to explore the possibility that peroxynitrite affects the characteristics of the y⁺ system, the predominant arginine uptake system. We found that in mesangial cells exposed to peroxynitrite (0.1 mM), the arginine transport system remained sodium independent. Excess concentration of lysine strongly inhibited L-arginine uptake while the neutral amino acid methionine had no effect (Figure 3). In addition, we characterized the kinetics of L-arginine transport by measuring saturable uptake of L-arginine (0–1 mM) in the presence of peroxynitrite (0.1 mM). The plots of L-arginine uptake as a function of extracellular L-arginine concentration are shown in Figure 4. A high affinity transporter was found to be present with a K_m of 135 μM. These data establish that in mesangial cells, exposed to peroxynitrite, system y⁺ remains the predominant arginine transport system with kinetic properties that resemble those of CAT-1 and CAT-2.

Regulation of mesangial CAT-1 and CAT-2 gene expression by peroxynitrite
To determine whether the observed peroxynitrite induced changes in arginine uptake are associated with similar directional changes in mRNA levels for the CAT family of transporters, northern blot analysis was performed. Mesangial cells exhibited a significant increase in the steady-state level of CAT-2 following exposure to peroxynitrite, while CAT-1 mRNA expression was significantly decreased (Figure 5).

Effect of peroxynitrite on mesangial CAT-1 protein
To further explore a possible effect of peroxynitrite on mesangial CAT-1, we examined CAT-1
protein levels. CAT-1 protein was identified as ~90 kDa. We found that CAT-1 abundance was low in untreated cells but further decreased following exposure to PN (Figure 6). Western blotting to CAT-2 protein was not performed due to failure to synthesize anti CAT-2 antibodies.

Discussion

In the present work, we demonstrate that exogenous peroxynitrite-related effects significantly stimulate arginine uptake by mesangial cells in primary cultures. This effect was concentration dependent up to a point where it started to decrease probably as a result of ONOO⁻ induced cell death. Our data are consistent with a previous finding by Vega-Agapito et al. [14], who reported increased arginine transport in glial cells subjected to peroxynitrite and suggested that this phenomenon serves to replenish arginine in neighbouring neurons.

There is one main question posed by these findings: what would be the consequences of increased arginine availability in ischaemic or inflamed glomeruli? Since mesangial cells function in proximity to the other cells that constitute the glomerulus, it is conceivable that this phenomenon may impact glomerular function. Indeed, reactive oxygen species generation by mesangial cells has been shown to decrease renal function [15]. One can hypothesize that the effect on arginine uptake initiates a destructive process by flooding tissues with excess reactive oxygen species.

Previous reports in the literature addressed these observations in a different manner. Xia et al. [16] have shown that cells grown in an arginine-depleted environment synthesize both NO and superoxide while cells cultured with adequate...
Peroxynitrite regulates arginine transport

Concentration of L-arginine generate only NO. Noris et al. [17] suggested that arginine depletion in pre-eclamptic human placentas orient nitric oxide synthase towards oxidant species. Moreover, arginine depletion plays a major role during ischaemia and reperfusion injury as there is a decrease in renal de novo arginine synthesis from citrulline and aspartate because of depleted adenosine 5'-triphosphate (ATP) levels, which further contributes to lower cytosolic levels of L-arginine [18,19]. In the aggregate, it appears that arginine depletion contributes to oxidative stress through the augmentation of oxygen free radical generation. The enhancement of arginine transport by the presence of peroxynitrite, as observed in our study, can serve as a protective mechanism limiting further peroxynitrite generation, thus attenuating tissue damage.

We have tried to elucidate a molecular mechanism to explain our findings. Among several transport systems that mediate L-arginine uptake (y+, B0+, B0,+ and y+ t), system y+ is considered to be the major arginine transporter in most tissues and cells. Encoded by cationic amino acid transporters CAT-1, CAT-2 and CAT-3, system y+ is characterized by a high affinity for cationic amino acids, sodium independence and stimulation of transport by substrate on the opposite (trans) side of the membrane [20,21]. Accumulated evidence from our laboratory and others suggest that each transporter has an affinity to a specific NOS isoform. It is believed that a specific NOS acquires its arginine from a specific transporter: eNOS by CAT-1 and iNOS by CAT-2 [11,22–24]. Therefore, regulation of a specific transporter by peroxynitrite could potentially enlighten an involvement of a specific NOS isoform in the pathogenesis of this complex induced cell toxicity.

The changes observed in arginine uptake, in our studies, were associated with differential regulation of CAT-1 and CAT-2. While CAT-2 mRNA was up-regulated, both CAT-1 mRNA and protein levels were significantly decreased—a conclusion that bears a limitation since we were unable to follow CAT-2 protein. Although RNA or protein levels do not necessarily correlate with the activity of the transporter, it seems conceivable to assume that peroxynitrite does have a negative impact upon CAT-1. We have previously reported that in the lipopolysaccharide (LPS)-induced acute renal failure model, increased arginine uptake and NO generation by CAT-2 and iNOS elicit an inhibitory effect on CAT-1 and eNOS [11,25]. The current experiment provide a possible mechanism to this phenomenon. High concentrations of peroxynitrite generated during ischaemic or inflammatory conditions elicit a selective inhibitory effect on the constitutive NO system, namely CAT-1 and eNOS. These conclusions must be dealt with caution since the abundance of CAT-1 protein in mesangial cells is small and the physiological role of eNOS in mesangial cells is unknown. Similar experiments should be repeated in endothelial cells.

In summary, we have shown that, in mesangial cells, effects induced by peroxynitrite augment arginine uptake via up-regulation of CAT-2. It appears likely that the excess arginine serves to attenuate further oxidative stress and limit tissue damage. At the same time, a possible inhibitory effect on CAT-1 may enhance cytotoxicity via inhibition of constitutive NO generation.

Conflict of interest statement. None declared.

References

22. McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric oxide synthase may explain the “arginine paradox”. J Biol Chem 1997; 272: 31213–31216

Received for publication: 11.4.06
Accepted in revised form: 7.8.06