Differential Effects of NO Inhibition in Renal Epithelial and Endothelial Cells in Mono-Culture vs. Co-Culture Conditions

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Key Words

NO • NOS • 4-ABH₄ • VAS203 • L-NMMA • Bilayer • HK-2 cells • EA.hy 926 cells

Abstract

Nitric oxide (NO) plays a critical role in the regulation of renal hemodynamics and tubular function after post-ischemic damage or sepsis. Diminished NO bioavailability contributes to endothelial dysfunction and may be caused by reduced NO synthesis due to substrate or co-factor deficiency. The aim of this study was to investigate the effects of NOS inhibition and NO depletion in a renal endo-epithelial bilayer model compared to monolayers of proximal tubular epithelial (HK-2) cells and endothelial cells of venous origin (EA.hy 926) with respect to cellular integrity, apoptosis and cytokine release. Two different NOS inhibitors have been used: an arginine-based-inhibitor, L-N^Gmonomethyl-arginine (L-NMMA) and a cofactor-based-inhibitor, H4-amino-biopterin (4-ABH₄) showing iNOS selectivity. We found significantly higher basal NO production by epithelial than by endothelial monolayers, which was significantly reduced by both NOS-inhibitors with a stronger effect demonstrated by 4-ABH₄. Furthermore we detected significant basal

iNOS protein expression in unstimulated HK-2 cells. NOS inhibition by 4-ABH₄ was associated with increased LDH release, apoptosis and reduced IL-6 production in epithelial but not in endothelial monolayers. These effects on epithelial cells were abolished under co-culture conditions. In contrast, endothelial cells showed higher IL-6 and IL-8 release under co-culture conditions than in monolayers, with IL-8 production being largely suppressed by L-NMMA but not by 4-ABH₄. In conclusion, inhibition of basal NO production in epithelial monolayers shows detrimental effects on cell integrity and viability. Under co-culture conditions interrelation between epithelial and endothelial cells appears to counteract these potentially harmful effects of epithelial NOS inhibition.

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Introduction

Nitric oxide (NO) is a short-living biological mediator that is regarded as one of the most important molecules in biology, due to its important role in many physiological processes, including neurotransmission, vasodilation, inflammation, autoimmunity, and pathophysiological conditions such as cardiovascular diseases and acute

kidney injury (AKI). NO enzymatically derives from the oxidation of the guanidino nitrogen of L-arginine by the activity of NO synthase (NOS) which exists in three different isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoform [1, 2]. All isoforms of NOS require NADPH, FAD, FMN, heme and tetrahydro-L-erythrobiopterin (H4biopterin) as cofactors. The source of NO in the kidney was originally thought to be exclusively the endothelial cells [1], but several studies indicate that tubular epithelial cells are capable of NO synthesis via both constitutive and inducible isoforms of NOS [3-5]. In contrast to many organs iNOS appears to be constitutively expressed in the kidney [6, 7]. In humans a relationship between the upregulation of renal iNOS and proximal tubular injury during systemic inflammation has been demonstrated [8]. Activation of iNOS during sepsis or ischemia-reperfusion leads to elevated NO levels that influence renal hemodynamics and cause peroxynitrite-related tubular injury through the local generation of reactive nitrogen species [1, 9-11]. Consequently, selective iNOS inhibition has been successfully applied in experimental models of these disease entities [5, 12].

However, not only increased NO production can be detrimental, also reduced NO bioavailability is implicated in the development of vascular diseases and may have profound effects at cellular level. Reduced NO synthesis, caused either by decreased levels of substrate (L-arginine) or by increased endogenous NO inhibitors such as asymmetric dimethylarginine (ADMA), and enhanced NO degradation triggered by oxidative stress potentially contribute to endothelial dysfunction. These alterations are very frequent in patients with chronic kidney disease (CKD) and may contribute to accelerated progression of CKD, hypertension and cardiovascular complications [13]. On cellular level reduced NO production may impair mitochondrial signaling necessary for the induction of cellular defense mechanism as adaptive responses to stress [14] and alter nephron transport [15]. As a consequence, NO was given the nickname of “vital poison” for the metabolic, immune and inflammatory pathways and a detailed understanding about how NO bioavailability is affecting the kidney at a cellular level is required.

To date, all *in vitro* studies concentrated on the (patho)physiology of NO in single monolayers (endothelial or epithelial cells). However, the situation *in vivo* is more intricate. Epithelial and endothelial cells form a complex network of interaction, and cooperate in solute and water re-absorption, secretion, and inflammation. The onset and progression of impaired renal function is based on a

defective interplay between the tubular and vascular renal compartments. To investigate these complex interactions, we recently established a co-culture model composed of human endothelial cells and human renal proximal tubular cells cultured on opposite sides of polycarbonate growth membranes, which mimics the renal interstitial situation [16, 17].

The aim of this study was to evaluate effects of arginine based (L-N^Gmonomethyl-arginine, L-NMMA) or cofactor based (H4-amino-biopterin, 4-ABH₄) NOS inhibitors and consequent NO depletion in a renal bilayer model compared to epithelial and endothelial monolayers with respect to cellular integrity, apoptosis and cytokine release.

Materials and Methods

Cell culture

HK-2 cells (human renal epithelial cell line with features of proximal tubular cells) [18] were obtained from the American Type Culture Collection (ATCC, Rockville, USA). Culture medium was a mixture of DMEM and Ham's F-12 medium (GIBCO-Invitrogen, Lofer, Austria) supplemented, with 10 ng/ml human recombinant epidermal growth factor (EGF), 36 ng/ml hydrocortisone, 5 µg/ml bovine insulin, 5 µg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM Glutamax (GIBCO), 100 U/ml penicillin, and 100 µg/ml streptomycin.

EA.hy 926 cells show characteristics of differentiated endothelial cells of venous origin, obtained by fusion of HUVECs with a bronchial carcinoma cell line [19]. EA.hy 926 cells were maintained in MCDB-131 medium supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.2 µg/ml hydrocortisone, and 10 ng/ml EGF.

Experimental design

Co-culture setup. The epithelial-endothelial bilayer was constructed according to Bijuklic et al., with slight modifications [16, 20]. Briefly, EA.hy 926 cells were seeded on the lower side of Transwell growth support (12 mm diameter, 0.4 µm membrane pore size; Corning, Germany). After EA.hy 926 cells reached confluence (2–3 days, as examined by microscopic analysis), HK-2 cells were seeded at $\sim 1 \times 10^5$ cells/ml into the upper compartment. Cells were considered to have reached confluence when they exhibited a transepithelial electrical resistance (TEER) higher than 17 Ωcm². TEER was measured with the Endohm system (World Precision Instr., Berlin, Germany). TEER of blank filters was subtracted. A model of the system is depicted in Figure 1.

The conditions and the treatments were kept unchanged for all the assays performed, except for western blot and immunofluorescence experiments. HK-2 and EA.hy 926 cell monolayers have always been incubated for 4 hours with

Fig. 1. Schematic description of the endo-/epithelial co-culture (bilayer) system. EA.hy 926 cells were seeded on the lower side of a Transwell growth support (12 mm diameter, 0.4 μ m membrane pore size), and HK-2 cells into the upper compartment. In the inset, particular of the filter chamber with cells seeded on it.

different concentrations (0, 100, 1000 μ M) of L-NMMA, an arginine-based NOS inhibitor, and 4-ABH₄, a tetrahydrobiopterine; cofactor based NOS inhibitor (VAS203, Vasopharm, Germany). HK-2/EA.hy 926 bilayers were incubated for 4 hours with 0, 100, 1000 μ M of the two different inhibitors (4-ABH₄ and L-NMMA) at the endothelial side of the transwell.

NO quantification assay

NO production by the cells of our three different models was quantified using the Griess Method, that measures the amount of nitrite as oxidized product of NO.

Supernatants of pre-treated cells (as indicated above) have been collected and assayed for the NO colorimetric assay (Biovision, USA), following manufacturer's instructions. Nitrite concentrations were determined using a multiwell plate reader (TECAN GENios; TECAN, Grödig, Austria).

iNOS expression

Western Blot. Expression of iNOS proteins in constitutive conditions was determined using Western blot according to Smith and Titheradge [21]. Briefly, HK-2 and EA.hy 926 cells were incubated for 4 hours with medium, 4-ABH₄ (1mM) or L-NMMA (1mM). Cells were washed and lysed with cold extraction buffer: 0.32M sucrose, 10mM Tris-HCl, 1mM EDTA, 1mM DTT, pH 7.5 supplemented with a protease inhibitor cocktail (complete mini EDTA free, Roche). To remove cell debris, cell homogenates were centrifuged at 30600xg at 4°C for 45 minutes using a Beckmann optima L-90K ultracentrifuge. Samples derived cell-lysates were quantified for protein content using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and the same amount of total protein (8 μ g) was loaded to each slot of a NuPAGE 4-12% Bis-Tris gels (Invitrogen, Lofer, Austria). Gels were then blotted onto PVDF and probed with a polyclonal Rabbit anti human iNOS IgG (Abcam, Wien, Austria). A HRP conjugated Goat Anti Rabbit IgG, (Millipore, Wien, Austria) was used as secondary antibody.

Fibronectin expression

Western Blot. Expression of total protein was determined using Western blot according to Burger et al [22]. Briefly, HK-2 and EA.hy 926 cells have been treated for 4 hours with medium (1), 150U/ml TNF α + 500U/ml IFN γ 2, 1mM 4-ABH₄ (3), 1mM L-NMMA (4). Cells were washed and lysed with cold cell-lysis buffer (PBS, 0.01 M EDTA, 0.001 M PMSF, 0.01% NaN₃, 1 mg/ml CHAPS). Cell lysates were then centrifuged at 10000 xg at 4°C for 15 minutes to remove cell debris.

Samples were quantified and equalized for protein content using the BCA Protein Assay Kit, electrophoresed on NuPAGE 4-12% Bis-Tris gels and then blotted onto PVDF. To detect fibronectin, polyclonal Rabbit anti-human Fibronectin IgG (Abcam, Wien, Austria), and HRP conjugated Goat Anti Rabbit IgG, were used.

Immunofluorescence microscopy

To detect and visualize fibronectin directly in HK-2 and EA.hy 926 cells, cells were grown in 6-well tissue plates. After 4 hours of stimulation with medium (1), 150U/ml TNF α + 500U/ml IFN γ (2), 1mM 4-ABH₄ (3), 1mM L-NMMA (4), supernatants were removed. Cells were fixed with 2% paraformaldehyde for 20 minutes, then permeabilized with 0.1 % Triton X-100 for 5 minutes, and blocking buffer (PBS + 10% FCS) was added for 1 hour at RT. To detect fibronectin, polyclonal Rabbit anti-human Fibronectin IgG, and Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Abcam, Wien, Austria) were used. Cells were also counterstained with DAPI (300nM). Images were acquired by an inverted microscope (Olympus IX70) equipped with a 20X objective (Olympus LCPlanFI) and appropriate filter sets for acquisition of DAPI and Alexa Fluor® 488 fluorescence. To allow for visual comparison, optical settings were kept constant for all images.

Cell vitality and Apoptosis

Cell vitality was determined by FACS (Fluorescence Activated Cell Sorter) analysis using Annexin-V and propidium iodide (PI). After treatment, cells were detached by a brief incubation with Trypsin-EDTA solution and then incubated with Annexin-V conjugated with fluorescein isothiocyanate (FITC) in binding buffer for 15 min, followed by PI staining (Human Annexin V-FITC kit, Bender MedSystem, Wien, Austria). The fluorescence intensity of apoptotic cells was measured with a flow cytometer (FACScalibur, Becton Dickinson) and compared to background controls using CELLQUEST software (BD Bioscience, Mississauga, ON).

Cell membrane damage

Cell membrane impairment was evaluated using a Cytotoxicity Detection kit (Roche Applied Science, Wien, Austria), which is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant.

Supernatants of pre-treated cells have been collected and the absorbance was read at 492nm using the TECAN GENios multiwell plate reader.

Fig. 2. Influence of 4-ABH₄ and L-NMMA on constitutive nitrite production in HK-2 and EA.hy 926 cells (monolayer and bilayers) and on iNOS expression. Nitrite release was measured in supernatants of HK-2 cells, EA.hy 926 cells monolayer (a) and in supernatants from the bilayer system (b) after 4 hours incubation with different concentration of 4-ABH₄ and L-NMMA. Data are represented as mean $\pm$ SEM of n=4 (each was a triplicate) for monolayers, and n=3 for bilayer. Inset. Western blot analysis of iNOS content in HK-2 and EA.hy 926 cells. Cell lysates were obtained from HK-2 cells and from EA.hy 926 cells previously treated with medium (1), 1mM 4-ABH₄ (2) or 1mM L-NMMA (3).

Cytokine secretion assay

Supernatants of pre-treated cells have been collected and assayed by ELISA (R&D Systems Inc. USA) for interleukin IL-6 and IL-8 according to the manufacturer's instructions.

Materials

All chemicals, except when mentioned, were from Sigma Chemical (Deisenhofen, Germany).

Statistical Analysis

The data are presented as mean $\pm$ standard error (SE) of at least three independent experiments, unless otherwise indicated. Each single experiment was always performed as triplicate. Differences between the means were tested for statistical significance with one-way Anova followed by Dunnett's multiple comparison test, or with Student's t-test, when applicable, using Graph Pad Software. P values of less than 0.05 were considered significant.

Results

Influence of NOS inhibitors on NO production in constitutive conditions

NO secretion by HK-2 cell, EA.hy 926 cell monolayer and from HK-2/ EA.hy 926 cell bilayers was quantified as amount of nitrite in the cell supernatants (Fig. 2a). HK-2 cells showed about 4 times higher amounts of nitrite release under basal conditions.

4-ABH₄ effectively decreased the amount of NO at each concentration used (100 μ M and 1mM), in epithelial ($7.78 \pm 1.15\%$ and $21.7 \pm 1.28\%$ reduction for 100 μ M and 1mM, respectively; n=4, p<0.01) and endothelial monolayers ($21.81 \pm 7.66\%$ reduction for 1mM; n=4, p<0.05). L-NMMA, instead, showed significant inhibition of NO release only in epithelial monolayers ($7.56 \pm 1.14\%$ and $8.46 \pm 2.16\%$ reduction for 100 μ M and 1mM, respectively; n=4, p<0.01). In cells of the bilayer system, the situation appeared different: endothelial cells directly exposed to the inhibitors showed a 50% decreased NO release after incubation with 1mM 4-ABH₄ (p<0.05 vs.

control), while NO release by epithelial cells remained unaffected.

Effect of NOS inhibitors on iNOS expression

To determine whether the effect of the selective and non selective NOS inhibitors on NO production was dependent on a direct effect on the iNOS expression, we performed western blot analysis.

Resting epithelial and endothelial monolayers showed a basal expression of iNOS (~ 130 kDa) as indicated in the insert in Figure 2a. The presence of 4-ABH₄ reduced the expression of the protein in both cells types; L-NMMA had a similar but less pronounced effect.

Effect of NOS inhibitors on electrochemical properties of the bilayer

TEER was measured routinely as parameter of confluence and integrity of the endo-epithelial bilayer system. To test the effect of NO inhibition on electrochemical properties of the bilayer, increasing

Fig. 3. Effect of NOS inhibitors on electrochemical properties of the bilayer. a) Measurement of transepithelial resistance (TEER) of bilayers, expressed as the delta value before and after 4 hours incubation with different concentrations of NOS inhibitors (n=3). b) Western blot and c) immunofluorescence of fibronectin expression in HK-2 and EA.hy 926 cells after incubation with medium (1), 150U/ml TNF α + 500U/ml IFN γ (2), 1mM 4-ABH $_4$ (3), 1mM L-NMMA (4). Data are representative of three separate experiments.

concentrations of 4-ABH $_4$ and L-NMMA were applied in the co-culture systems (Fig. 3a). The HK-2/ EA.hy 926 co-culture incubated with L-NMMA (on the bottom compartment, endothelial side) exhibited a concentration-dependent increase in electrical resistance ($p=0.02$, at 1mM); instead, there was no significant difference in electrical resistance of the bilayer when incubated with different concentrations of 4-ABH $_4$, indicating that electrochemical properties of the bilayer were not influenced by the inhibitor.

Effect of NOS inhibitors on fibronectin expression and release

To evaluate the potential involvement of matrix proteins as key-players in the TEER increase observed in presence of 1mM L-NMMA, we investigated the expression of fibronectin in HK-2 and EA.hy 926 cell monolayers. Western blot analysis (Fig. 3b) revealed a slight increase in fibronectin production in L-NMMA treated cells over control and 4-ABH $_4$ in cell lysates. Immunofluorescence staining even clearer demonstrated the increase in fibronectin production in L-NMMA treated cells. TNF α + IFN γ has been used as positive control for western blot and immunofluorescence analysis [16].

Effect of NOS inhibitors on cells vitality (and apoptosis)

To determine the effect of NO depletion on tubular epithelial and endothelial vitality, we performed FACS analysis on cells previously treated with selective and non-selective NO inhibitors. Vitality was generally decreased after 4 hours treatment with either 4-ABH $_4$ or L-NMMA in HK-2 cells cultured as monolayer, but it was statistically different from control values, only with 1mM 4-ABH $_4$ (58.00 ± 9.96 , $p<0.05$, $n=4$; Fig. 4a). Endothelial cells were never affected by the presence of NOS inhibitors (Fig. 4b). Furthermore, when epithelial and endothelial cells cultured as bilayer the percentage of apoptotic cells was comparable to control values (Fig. 4c). Apoptosis is in line with the results obtained for cell vitality (Fig. 4d-f), while necrosis was never significant in any of our

cell models (data not shown).

Effect of NOS inhibitors on cell membrane integrity

To better understand whether the reduction in cell vitality observed in HK-2 cells monolayers in presence

Fig. 4. Effect of NOS inhibitors on cell vitality (a, b and c) and cell apoptosis (d, e, and f) in HK-2 cell monolayer (a and d), EA.hy 926 cell monolayer (b and e) and bilayers (c and f). FACS analysis using Annexin V- FITC conjugated and PI staining in HK-2, EA.hy 926 cell monolayer and bilayers, treated for 4 hours with 4-ABH₄ or L-NMMA at different concentrations (100μM and 1mM). Results are represented as mean ± SEM of n = 4.

of 1mM 4-ABH₄ could be due to an effect on cell integrity, we analyzed LDH release after treatment with the selective and non selective inhibitors. Four hours of incubation of HK-2 cell and EA.hy 926 cell monolayers with 4-ABH₄ resulted in a four-fold and two-fold release of LDH, respectively, at the highest dose applied (1mM) as compared to controls ($p < 0.01$, $n = 7$ and $n = 6$, respectively), as indicated in Figure 5. The behavior of the cells of the bilayer was different: epithelial cells were not affected by the presence of 4-ABH₄ at any concentration used; endothelial cells of the bilayer directly in contact with the inhibitor, showed a small but distinctly higher release of LDH compared to controls, when treated with 1mM 4-ABH₄ ($p < 0.01$, $n=6$).

No difference in cell membrane integrity was observed between control and cells pre-treated with L-NMMA (100μM and 1mM), either in the monolayers either in the bilayer.

Effect of NOS inhibitors on cytokine release

To analyze potential effects of NO depletion on cellular inflammatory reaction we investigated the cellular

cytokines release in our three different models after treatment with NOS inhibitors. Interestingly, pre-treatment with 4-ABH₄ (1mM) showed significantly decrease IL-6 release ($53.06 \pm 12.99\%$, $p < 0.001$; $n=6$; Fig. 6a) in epithelial monolayers. Furthermore, as depicted in Figure 6f, L-NMMA incubation (1mM) resulted in 50% decreased IL-8 release by endothelial cell when in co-culture with the epithelial cells ($p < 0.01$, $n=6$; Fig. 6f). Notably, epithelial cells, clearly decreased their levels of cytokine release, when in co-culture with endothelial cells, while endothelial cells increased their release of cytokine when co-culture even under control conditions.

Discussion

The present study was carried out to evaluate the effect of NOS inhibition and consecutive NO depletion in a renal bilayer model compared to epithelial and endothelial monolayers with respect to cellular integrity, apoptosis and cytokine release. We found significantly

Fig. 5. Effect of NOS inhibitors on cell membrane impairment in HK-2 cell monolayer (a), EA.hy 926 cell monolayer (b) and bilayers (c). LDH release was measured in the supernatants of HK-2 and EA.hy 926 cells cultured as monolayer or as bilayer, after 4 hours of incubation with different concentrations of 4-ABH₄ and L-NMMA. a) LDH release in HK-2 cells cultured as monolayer; b) LDH release in EA.hy926 cells cultured as monolayer; c) LDH release in HK-2 and EA.hy926 cells cultured as bilayer. Results are represented as mean $\pm$ SEM of $n > 6$.

Fig. 6. Cytokine release in HK-2 cell monolayer (a), EA.hy 926 cell monolayer (b) and bilayers (c). Release of IL6 and IL8 was measured by ELISA in the supernatants of HK-2 and EA.hy 926 cells cultured as monolayers, or as bilayer, after 4 hours of incubation with the 4-ABH₄ and L-NMMA. (d) Cytokine release in HK-2 cells cultured as monolayer; (e) Cytokine release in EA.hy926 cells cultured as monolayer; (f) Cytokine release in HK-2 and EA.hy926 cells cultured as bilayer. Results are presented mean $\pm$ SEM of $n > 6$.

higher basal NO production by epithelial cells as compared to endothelial monolayers. Both unspecific NOS inhibition by L-NMMA and more selective iNOS inhibition by 4-ABH₄ significantly reduced NO release from

both endothelial and epithelial monolayers. Inhibition of NOS by 4-ABH₄ was associated with increased LDH release, apoptosis and reduced IL-6 production in epithelial but not in endothelial monolayers. These effects on

epithelial cells were abolished under co-culture conditions. In contrast, endothelial cells exhibited drastically increased IL-6 and IL-8 release under co-culture conditions as compared to monolayers with IL-8 production being largely suppressed by L-NMMA but not by 4-ABH₄.

NOS exists in three different tissue specific isoforms: endothelial (eNOS), neuronal (nNOS) and an inducible (iNOS) isoform [1, 2]. Classical NOS inhibitors, such as L-NMMA, are non selective arginine-based blockers. In contrast to L-NMMA, and all other currently available NOS inhibitors, 4-ABH₄ does not inhibit NOS at the L-arginine binding site but at the tetrahydrobiopterine (BH₄) cofactor binding site. This novel mechanism of action results in an improved specificity profile, i.e. 4-ABH₄ almost exclusively inhibits NO synthases and prevents reactive oxygen species production under conditions of oxidative stress by avoiding uncoupling of NOS [23-25]. Several groups already reported that, when purified, the three NOS isoforms are equally sensitive to 4-ABH₄, while in cultured cells stimulated by cytokines as well as in aortic strips there is a preferential inhibition of iNOS due to the faster turnover of iNOS protein as compared to eNOS [26, 27]. When applied to monolayers we observed an inhibitory effect of 4-ABH₄ on basal production of NO which was more pronounced in epithelial cells than in endothelial cells. This difference could be due to subtle structural differences in the catalytic sites of the epithelial and endothelial NOS isoforms. Moreover, 4-ABH₄ exhibited stronger attenuation of the basal production of NO than L-NMMA. Several explanations can be provided for this finding. First, this could be explained by a potential lack of an appreciable isoform selectivity of 4-ABH₄ *in vivo* in the absence of NOS induction, which is, however, not really substantiated by previous investigation in various cell types [26, 27]. Secondly, a difference in the concentration necessary to achieve the same reduction of activity when acting either on the substrate (L-NMMA) or on the cofactor (4-ABH₄) site on NOS may be assumed. Thirdly, NO production of HK-2 monolayers may predominantly result from iNOS activity. In concordance to other groups [6] protein expression analysis in our experiments revealed a high basal expression of iNOS in epithelial cells and a much lower expression in endothelial cells. Down-regulation induced by 4-ABH₄ was more pronounced than by L-NMMA. Thus, the reduction of nitrite release observed could be due to decreased levels of iNOS due to an inhibition of the dimerization induced by the 4-ABH₄ and, to a lesser extent, by L-NMMA. iNOS content appeared much higher in epithelial than in endothelial cells.

Consequently, the significantly higher NO production by HK-2 cells, deriving primarily from iNOS which appears to be constitutively higher expressed in epithelial than in endothelial cells appears as the most likely explanation.

In the bilayer model, the effect of 4-ABH₄ is still present in the endothelial cells being in direct contact with the drug, but it is not influencing the epithelial cells, which are not directly exposed. However, also nitrite release from epithelial cells was drastically reduced when cultured together with endothelial cells.

With respect to cellular integrity and vitality, 4-ABH₄ at a high dose (1mM) exerted a negative effect on membrane integrity of tubular epithelial cells indicated by a significant LDH release (Fig. 5a) associated with dose dependent decrease on cell vitality (Fig. 4a) and increased apoptosis (Fig. 4d). Although endothelial monolayers also showed slightly increased LDH release in the presence of 4-ABH₄, this was apparently without consequences on cellular viability.

Evidence from other groups indicate that increased NO production in epithelial cells is protective against damage in post-ischemic conditions [28]. But even under physiologic conditions NO apparently plays an essential role in mitochondrial signaling necessary for the induction of cellular defense mechanism as adaptive responses to stress [14]. NO interaction with cytochrome C oxidase in different cell types is associated with the resistance to apoptosis induced by various kinds of stressors like growth factor deprivations or intracellular calcium overload [29, 30]. Therefore NOS inhibition may be considered detrimental especially for renal epithelial cells under basal condition (unstimulated monolayers) by reducing protective intracellular and mitochondrial NO levels.

The harmful effect of 4-ABH₄ on epithelial monolayers appears to be partially abolished, when the epithelial and endothelial cell types are cultured together in a bilayer system: endothelial cells, directly in contact with the inhibitor, are still affected, but epithelial cells are protected from the damage by the endothelial barrier. The significantly reduced nitrite release of HK-2 cells when co-cultured with endothelial cells may indicate reduced stressors. Under this condition NOS inhibition may be less detrimental.

It has to be admitted, however, that injurious and pro-apoptotic effects of 4-ABH₄ could be also related to formation of hydrogen peroxide due to auto-oxidation of the compound itself, independent from NO production [31]. The lack of effect of L-NMMA either on cell membrane impairment nor on cell vitality in any of our

models could support this hypothesis (Fig. 4-5). Based on the lower effects of L-NMMA on nitrate production, however, it is likely that L-NMMA simply was not as effective in iNOS inhibition as 4-ABH₄ when applied at the same concentration as already mentioned in the paragraph above.

Cytokine production, specifically IL-6 or IL-8, was evaluated as sign of inflammatory reaction. Both IL-8 and, to a larger extend, IL-6 release were already much higher in epithelial than endothelial cells monolayers under control conditions. Co-culturing of epithelial and endothelial cells resulted in opposite changes: significantly reduced epithelial cytokine release associated with an increased endothelial IL-8 but also IL-6 production. Parallel to its pro-apoptotic effects 4-ABH₄ attained a more than 50% reduction of IL-6 release by HK-2 monolayers, which was abolished in the co-culture conditions. Reduced pro-inflammatory cytokine production achieved by NOS inhibition has already been reported by other groups using various injury models [32, 33] whereas other report down regulation of cytokine production by increased NO [34]. The mechanism by which NO can affect cytokine production is at present unknown.

When determining transepithelial electrical resistance of the endo-epithelial bilayer we found a dose- dependent increase induced by L-NMMA but not by 4-ABH₄. Consequently, we evaluated the possibility that this increase could be due to an overexpression of proteins involved in cell-matrix or cell-cell interaction. We found associated increased fibronectin expression in epithelial cells as reported by other groups [22, 35]. It is likely that fibronectin, a major determinant of adhesion properties as well as a constituent of extra cellular matrix, does interact with the integrine family of transmembrane receptors [35] and therefore enhances the TEER, as previously demonstrated by Sugahara and co-workers when

fibronectin was used as substrate [36]. In EA.hy 926 cells we observed an enhancement of fibronectin production induced by iNOS activation (stimulation with TNF α and IFN γ , Fig. 3b), as previously reported [37], while the presence of the NOS inhibitors induced a slight decrease of fibronectin expression. The comparison with the epithelial cells led us to an interesting finding: L-NMMA-treated HK-2 cells exhibited enhanced levels of fibronectin, similar to those obtained in cells treated with TNF α and IFN γ (Fig. 3b). A possible reason for this behaviour could lie in the mechanism of action of L-NMMA, that, differently from 4-ABH₄, interferes with the arginine metabolism, which is known to modulate net protein synthesis in several cell types [38], and, thus, could be part of an alternative mechanism for fibronectin induction, that does not involve NO.

In conclusion, in contrast to endothelial cells, iNOS inhibition by 4-ABH₄ may be detrimental for renal epithelial cells in non-injurious but less physiological conditions, i.e. when cultured in monolayers, resulting in membrane damage, increased apoptosis and reduced cytokine production. When cultured in more physiological conditions, such as in the bilayer system, almost all the harmful effects of the NOS inhibitors are abolished possibly by the interrelation between the two cell types, the molecular mechanism of which could be matter of future investigations.

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