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L-Arginine supplementation prevents intestinal epithelial barrier breakdown under heat stress conditions by promoting nitric oxide synthesis

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ABSTRACT

Heat stress (HS) induced by exposure to high ambient temperatures or prolonged excessive physical activities is known to primarily induce deleterious effects on the intestinal integrity by disrupting junctional complexes. Considering the association of L-arginine (L-Arg) with the improvement of gut function, the hypothesis of this study was to assess whether L-Arg supplementation can prevent the intestinal barrier disruption under HS conditions and to understand whether the L-Arg–induced effects are associated with maintaining nitric oxide (NO) as the major product of L-Arg metabolism. For this study, human colorectal adenocarcinoma (Caco-2) cells grown on Transwell inserts were pretreated with different L-Arg concentrations (0.4, 1, and 4 mmol/L), and after exposure to HS, markers of intestinal barrier integrity, stress-related markers, and NO levels were determined. L-Arg deprivation markedly increased the mRNA expression of heat shock protein 70 and heme-oxygenase-1 under HS conditions. The HS-induced drop in transepithelial electrical resistance values and increase in Lucifer Yellow permeability could be prevented by 4 mmol/L L-Arg supplementation. In turn, L-Arg mitigated the downregulation and delocalization of adherens junction protein E-cadherin in HS-exposed cells. NO and inducible NO synthase levels were significantly decreased in HS-exposed cells, whereas pretreatment with 4 mmol/L L-Arg prevented this decrease. Inhibition of inducible NO synthase by the NO synthase inhibitor L-NG-nitroarginine methyl ester abrogated the effect of L-Arg on preserving intestinal integrity under HS conditions as measured by transepithelial electrical resistance, Lucifer Yellow flux, and E-cadherin expression. In summary, L-Arg supplementation protects the intestinal epithelial integrity, at least partly, by maintaining NO synthesis under HS conditions.

Abbreviations: AJ, adherens junctions; ANOVA, analysis of variance; Caco-2, epithelial colorectal adenocarcinoma; DMEM, Dulbecco modified Eagle minimum essential medium; HO-1, heme oxygenase-1; HS, heat stress; HSP70, heat shock protein 70; iNOS, inducible nitric oxide synthase; L-Arg, L-Arginine; L-NAME, L-NG-nitroarginine methyl ester; LY, Lucifer Yellow; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; qRT-PCR, quantitative real-time polymerase chain reaction; PBS, phosphate-buffered saline; TEER, transepithelial electrical resistance; TJ, tight junctions.

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1. Introduction

L-Arginine (L-Arg), classified as a semiessential amino acid, is involved in different physiological functions. The endogenous synthesis of L-Arg depends on cell type, age, developmental stage, diet, injuries, and disease status [1, 2]. Previous reviews have addressed the clinical potential of L-Arg supplementation, which has been shown to improve reproductive, cardiovascular, pulmonary, renal, gastrointestinal, liver, and immune functions. Its therapeutic applications also include patients with obesity, diabetes, and metabolic syndromes [2-4]. L-Arg serves as a precursor of physiologically important molecules, such as polyamines, creatine, agmatine, and most importantly nitric oxide (NO) [1]. The synthesis of NO is mediated by one of the constitutively expressed isoforms of nitric oxide synthase (NOS), such as neuronal NOS, endothelial NOS, or its inducible form inducible nitric oxide synthase (iNOS), which is expressed in different cell types [5]. In the gastrointestinal tract, basal iNOS activity and NO production are required for tolerance to stress conditions, whereas excessive NO levels are destructive by enhancing epithelial cell apoptosis [6]. Tanaka et al demonstrated that the protective effect of NO in the intestine is associated with increased mucus and fluid secretions as well as an inhibition of intestinal hypermotility [7]. Moreover, L-Arg–induced NO production has been introduced as a measure to mitigate inflammatory responses and to improve the intestinal integrity in experimental models of inflammatory bowel disease and hypoxia [8, 9]. In mice, it is shown that, after intestinal obstruction, the L-Arg–induced NO production can also reduce the bacterial translocation by increasing the levels of the IL-10 anti-inflammatory cytokine and secretory immunoglobulin A [10].

Exposure to high ambient temperatures, strenuous exercise, or their combination can lead to heat stress (HS) that can progress into heat stroke, which is considered as a life-threatening condition affecting the physiological function of different organs, including the intestines [11, 12]. Various in vitro and in vivo investigations have described that HS can disrupt the intestinal integrity [13-16]. We and others previously could show that this HS-induced intestinal barrier dysfunction is associated with alterations in the expression and cellular localization of tight junction (TJ) and adherens junction (AJ) proteins. A functional junctional complex is needed to seal the paracellular space between adjacent cells, thereby preventing the penetration of luminal antigens and microbes, including pathogens [15, 17, 18]. Especially, the E-cadherin expression and localization in the Caco-2 monolayer are targeted by HS, as published by our group before [15].

Previously, Costa et al demonstrated that dietary supplementation with L-Arg is an effective intervention strategy to maintain the intestinal barrier integrity in mice forced to physical exercise under environmental HS [19]. Moreover, other findings demonstrate the impact of L-Arg on preventing intestinal damage, modulating mucosal immunity, and reducing bacterial translocation across the gut barrier [20-22]. However, there is limited information about the various mechanisms by which L-Arg preserves the gastrointestinal barrier function, in particular under HS conditions. Considering the deleterious effect of HS on intestinal epithelium [15, 17] and previous findings suggesting the impact of L-Arg on intestinal function, the hypothesis of this study was to assess (1) whether L-Arg supplementation can prevent intestinal barrier disruption under HS conditions and (2) whether the L-Arg–induced effect is related to preserving NO levels because L-Arg serves as a precursor of NO. To test this hypothesis, firstly, the intestinal barrier integrity (transepithelial electrical resistance [TEER], Lucifer Yellow [LY] flux, E-cadherin expression and distribution), oxidative stress induction (heat shock protein 70 [HSP70] and heme oxygenase-1 [HO-1]), and NO production in epithelial colorectal adenocarcinoma (Caco-2) cells exposed to HS with and without pretreatment with L-Arg were examined. Secondly, the importance of NO in protecting the intestinal barrier function in this Caco-2 cell model was investigated by inhibiting iNOS using L-NG-nitroarginine methyl ester (L-NAME), the NOS inhibitor.

2. Methods and materials

2.1. Cell culture

Caco-2 cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA; passages 5-19) and were cultured as a monolayer in Dulbecco modified Eagle minimum essential medium (DMEM) supplemented with 25 mmol/L HEPES, 4.5 g/L glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 10% inactivated fetal calf serum (Gibco, Invitrogen, Carlsbad, CA, USA), glutamine (2 mmol/L, Gibco, Invitrogen, Carlsbad, CA, USA), 1% nonessential amino acids, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2, as described previously [15]. For all experiments, Caco-2 cells were seeded on 0.3-cm^2 high–pore density polyethylene terephthalate membrane Transwell inserts with 0.4-μm pores (Falcon, BD Biosciences, San Diego, CA, USA) placed in 24-well plates (0.3×10^5 cells/Transwell insert). All Transwell experiments started after obtaining a confluent Caco-2 monolayer at day 17-19 of culturing with transepithelial electrical resistance (TEER) values in the range of 400 $\Omega.cm^2$.

2.2. L-Arg and/or L-NAME pretreatment and HS exposure

After obtaining differentiated confluent Caco-2 monolayers at day 17-19 of culturing, culture medium was replaced by L-Arg–free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with antibiotics (penicillin 100 U/mL and streptomycin 100 μg/ml) (Gibco, Invitrogen, Carlsbad, CA, USA) and different noncytotoxic concentrations of L-Arg (0 mmol/L [L-Arg deprivation], 0.4 mmol/L [standard medium concentration], 1 mmol/L, or 4 mmol/L) (Sigma-Aldrich, St. Louis, MO, USA) [9]. After 24 preincubation with different concentrations of L-Arg, Caco-2 cells were subjected to control (37°C) or HS (42°C) conditions in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours without changing the media [15, 23].

For iNOS inhibition studies, 12 mmol/L L-NAME (Sigma-Aldrich, St Louis, MO, USA) was added to Caco-2 monolayers (apical and basolateral) 1 hour prior to incubation with

different concentrations of L-Arg (0, 0.4, and 4 mmol/L) for 24 hours (in total, 25 hours of L-NAME incubation). The used L-NAME concentration was based on previous data showing that at least 3 times more L-NAME than L-Arg is required for a competitive inhibition of iNOS [9].

2.3. Cell viability assay

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were seeded on 96-well plates and grown for 17 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to the experiment, cells were treated with 100 μL L-Arg–free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with different concentrations of L-Arg and/or L-NAME and exposed to control or HS conditions for 24 hours. Four hours prior to the end of HS exposure, 20 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (5 mg/mL phosphate-buffered saline [PBS]) was added to each well according to manufacturer's instruction. Thereafter, the cell medium was discarded, and 50 μL of DMSO was added to lyse the cells. Absorbance values were measured at 595 nm (FLUOstar OPTIMA, Offenburg, Germany). Neither HS exposure nor L-Arg and/or L-NAME pretreatment in the used concentrations affected the cell viability (Supplemental Fig. S1).

2.4. TEER measurement

The integrity of the intestinal epithelial monolayer was assessed by measuring TEER using a Millicell-ERS Voltohmmeter (Millipore, Temecula, CA, USA) as previously described [24]. Average TEER values of established Caco-2 cell monolayers prior to the start of the experiment were in the range of 400 ± 30 $\Omega.cm^2$. TEER was measured prior to and 24 hours after HS exposure, and results are expressed as a percentage of the initial value.

2.5. Paracellular tracer flux assay

The paracellular permeability was investigated by measuring the transfer of LY (0.457 kDa, 20 μg/mL) (Sigma-Aldrich, St Louis, MO, USA) across an established Caco-2 monolayer according to a previously described protocol [15]. Four hours prior to the end of 24-hour HS challenge, LY was added to the apical compartment of the Transwell inserts. At the end of HS challenge, the medium from the basolateral compartment was collected, and the fluorescence intensity of LY was measured by a fluorometer (FLUOstar OPTIMA, Offenburg, Germany) at excitation and emission wavelengths of 410 nm and 520 nm, respectively. The amount of LY flux was determined based on the LY calibration curve (0 to 100 μg/mL).

2.6. RNA isolation and quantitative real-time polymerase chain reaction

At the end of the experiment, Caco-2 cells were collected for RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR) analysis according to previously described protocols [15]. Briefly, cells were washed twice with ice-cold PBS and were lysed by adding 100 μL RNA lysis buffer containing β-mercaptoethanol. Total RNA was isolated using spin columns (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA levels and purity were measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) with a A_{260}/A_{280} ratio between 1.8 and 2. cDNA synthesis was performed using iScript cDNA Synthesis kit (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to manufacturer's instructions. The qRT-PCR analysis was conducted to assess the mRNA expression of arginase II, HSP70, and HO-1 by using iQSYBR Green Supermix and CFX96 C1000 Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA, USA). Supplemental Table S1 represents the commercially manufactured sets of gene-specific primers (Eurogentec, Seraing, Belgium) and the corresponding annealing temperatures, which were used after confirmation of specificity and efficiency analysis by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55°C to 65°C) for primer annealing and subsequent melting curve analysis. The mRNA quantities of the target genes were calculated as fold change of gene expression relative to untreated (control) cells and normalized to the β-actin reference gene.

2.7. NO measurement

Because, in an aqueous environment like cell culture medium, NO is rapidly converted into the stable end products, nitrite and ultimately to nitrate, nitrite was measured by the Griess reaction as reported previously [25]. Nitrite levels were analyzed in the apical compartment of Transwell inserts due to the abundant apical secretion by polarized intestinal epithelial cells [26]. Briefly, 100 μL of culture medium from the apical compartment of Transwell inserts was mixed with an equal volume of Griess reaction mix composed of 1% sulfanilamide (Sigma-Aldrich, St Louis, MO, USA) and 0.1% N-(1-naphthyl)ethylenediamine (Sigma-Aldrich, St Louis, MO, USA) in 5% H_3PO_4. The mixture was incubated for 5 minutes at room temperature, and absorbance was assessed at 540 nm (FLUOstar OPTIMA, Offenburg, Germany) and compared with a sodium nitrite standard curve.

2.8. Western blot analysis

Caco-2 cells were lysed using 50 μL RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Pennsburg, Germany). Total protein concentrations were measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions as described previously [16]. Equal protein amounts of samples were separated by electrophoresis (Criterion Gel, 4%-20% Tris-HCL, Bio-Rad Laboratories Inc, Hercules, CA, USA) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, the Netherlands). Membranes were blocked with PBS supplemented with 0.05% Tween-20 and 5% milk proteins and incubated overnight at 4°C with antibodies against iNOS (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) or E-cadherin (1:1000, eBioscience, San Diego, CA, USA), and β-actin (1:4000; Cell Signaling, Beverly, MA, USA) for equality of sample loading. Membranes were incubated with ECL Prime western blotting Detection Reagent (Amersham Biosciences, Roosendaal, the Netherlands) prior to obtaining the digital

images with the ChemiDoc MP imager (Bio-Rad Laboratories Inc, Hercules, CA, USA). The ImageJ 1.47 software was used to quantify the signal intensity of western blot bands. The protein levels were normalized with β-actin and are expressed as mean fold change in comparison with the control group.

2.9. Immunofluorescence staining

Caco-2 cells were grown on inserts and treated as mentioned above. As previously described [15], the inserts with Caco-2 cells were fixed with 10% formalin for 10 minutes, washed with PBS, and permeabilized with PBS containing 0.1% Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% bovine serum albumin/PBS for 30 minutes at room temperature. Thereafter, Caco-2 cells were incubated (2 hours at room temperature) with primary antibodies against iNOS (1:100, Thermo Fisher Scientific, Waltham, MA, USA) and E-cadherin (1:50, BD Biosciences, San Diego, CA, USA) followed by incubation with Alexa-Fluor–conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). After performing a nuclear counterstaining with Hoechst 33342 (1:2000, Invitrogen, Carlsbad, CA, USA), the inserts were mounted with FluorSave Reagent (Calbiochem, Schwalbach, Germany), and immunolocalization of iNOS and E-cadherin was determined using a Nikon Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) equipped with a Nikon Digital Sight DS-U1 camera (400×).

2.10. Statistical analyses

Analyses were performed by using GraphPad Prism (version 6.0) (GraphPad, La Jolla, CA, USA). Experimental results are expressed as means ± SEM of 3 independent experiments each performed in triplicate based on previous studies [15, 23]. Differences between groups were statistically determined by using 2-way analysis of variance (ANOVA) with Bonferroni post hoc test. Results were considered statistically significant when $P < .05$.

3. Results

3.1. L-Arg pretreatment prevents the HS-induced disruption of the intestinal epithelial integrity

Exposure of Caco-2 cells to HS for 24 hours induced a disrupted intestinal epithelial barrier observed by a drop in TEER values and increased LY permeability across the intestinal monolayer. Pretreatment with 4 mmol/L L-Arg significantly restored the HS-induced decrease in TEER values compared to lower L-Arg concentrations (0, 0.4, and 1 mmol/L) (Fig. 1A). Pretreatment with L-Arg concentrations showed a trend (not significant) in preventing the HS-induced increase in LY permeability (Fig. 1B).

3.2. L-Arg deprivation modulates the mRNA expression of HSP70 and HO-1

Exposure to HS for 24 hours resulted in an upregulation in the HSP70 (Fig. 2A) and HO-1 (Fig. 2B) mRNA expression in Caco-2 cells. This increase was more pronounced in the absence of L-Arg (0 mmol/L L-Arg) compared to L-Arg supplementation groups. The L-Arg deprivation resulted in an approximately 120-fold increase in HSP70 mRNA expression, whereas cellular HO-1 mRNA expression was increased about 20-folds under HS conditions. No significant differences were found between the effects of the different L-Arg concentrations on the HS-induced HSP70 and HO-1 mRNA expression (Fig. 2A and B).

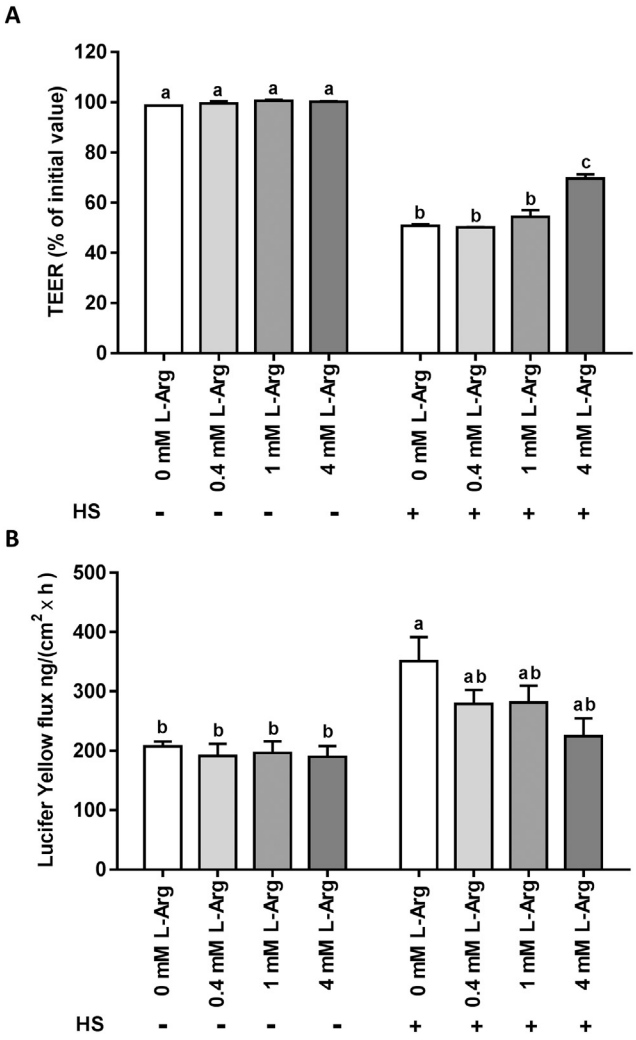

Fig. 1 – L-Arg partly prevents the disruption of intestinal integrity under HS conditions. Caco-2 cells grown on inserts were pretreated with different concentrations L-Arg (0, 0.4, 1, and 4 mmol/L) for 24 hours prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours. TEER levels (A), as well as LY transport (B) across the Caco-2 monolayer, were measured. Results are expressed as a percentage of initial TEER value (A) and the amount of tracer transported [ng/(cm² × hours)] (B), as means ± SEM of 3 independent experiments each performed in triplicate. For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used. Different lowercase letters denote significant statistical differences among groups ($P < .05$).

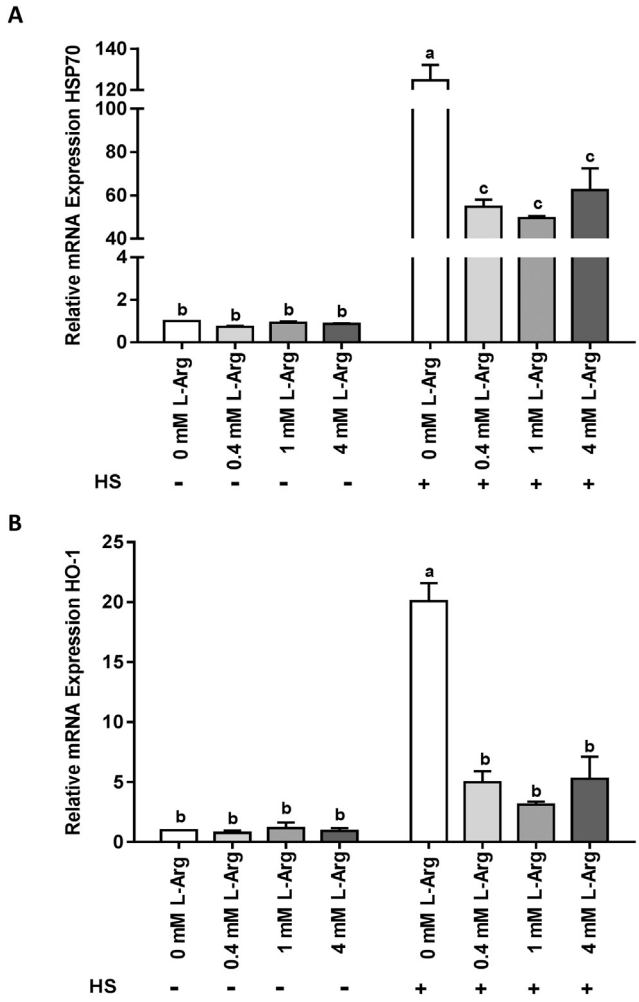

Fig. 2 – L-Arg deprivation markedly increases the mRNA expression of HSP70 and HO-1. Caco-2 cells grown on inserts were pretreated with different concentrations L-Arg (0, 0.4, 1, and 4 mmol/L) L-Arg for 24 hours prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours. HSP70 (A) and HO-1 (B) mRNA expression was assessed by qRT-PCR, and results are expressed as relative mRNA expression normalized to ß-actin (A, B) as means ± SEM of 3 independent experiments each performed in triplicate. Different lowercase letters denote significant statistical differences among groups ($P < .05$). For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used.

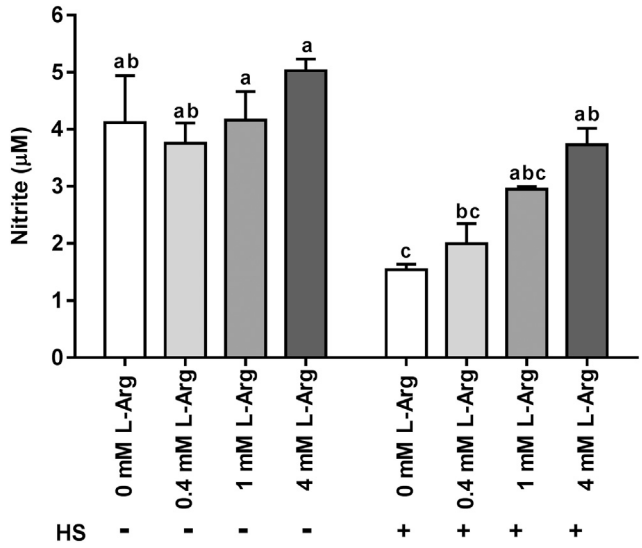

Fig. 3 – Supplementation with L-Arg prevents the HS-induced decrease in NO production. Caco-2 cells grown on inserts were pretreated with different concentrations of L-Arg (0, 0.4, 1, and 4 mmol/L) for 24 hours prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours. Production of nitrite, a stable metabolite of NO, was measured by a Griess reaction. Results are expressed as means ± SEM of 3 independent experiments each performed in triplicate. For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used. Different lowercase letters denote significant statistical differences among groups ($P < .05$).

3.3. *L-Arg supplementation prevents the HS-induced decrease in NO production*

Measurement of the NO production (by means of nitrite) revealed that exposure to HS significantly decreased NO release at the apical compartment. Supplementation of L-Arg increased the measurable nitrite concentration under control and HS conditions in a concentration-dependent manner. Under HS conditions, the increase in nitrite induced by 4 mmol/L L-Arg was significantly different compared to the corresponding control group, and an almost complete restoration of NO levels was achieved as compared to the Caco-2 cells under control conditions (Fig. 3).

3.4. *L-Arg–induced iNOS production prevents the HS-related disruption of intestinal integrity*

As shown in Fig. 4A, pretreatment of Caco-2 cells with 4 mmol/L L-Arg prior to HS exposure significantly prevented the HS-induced TEER decrease compared to L-Arg–free medium and medium containing 0.4 mmol/L L-Arg. This effect of L-Arg was abolished after inhibition of iNOS by L-NAME (Fig. 4A). In addition, inhibition of iNOS even exacerbated the HS-induced TEER decrease in Caco-2 cells treated with different L-Arg concentrations (0, 0.4, and 4 mmol/L L-Arg).

The HS-induced TEER drop was accompanied by an increased transfer of LY across the intestinal epithelial monolayer. Pretreatment with 4 mmol/L L-Arg significantly prevented the increased LY flux under HS conditions, whereas inhibition of iNOS with L-NAME aggravated the HS-induced increase in paracellular (LY) permeability in Caco-2 exposed to different L-Arg concentrations (0, 0.4, and 4 mmol/L L-Arg) (Fig. 4B).

3.5. *L-Arg promotes the expression of E-cadherin and prevents the HS-induced downregulation and delocalization of E-cadherin*

Under thermal neutral conditions, cells treated with L-Arg or L-NAME did not show any remarkable changes in the expression of E-cadherin in protein levels. However, under

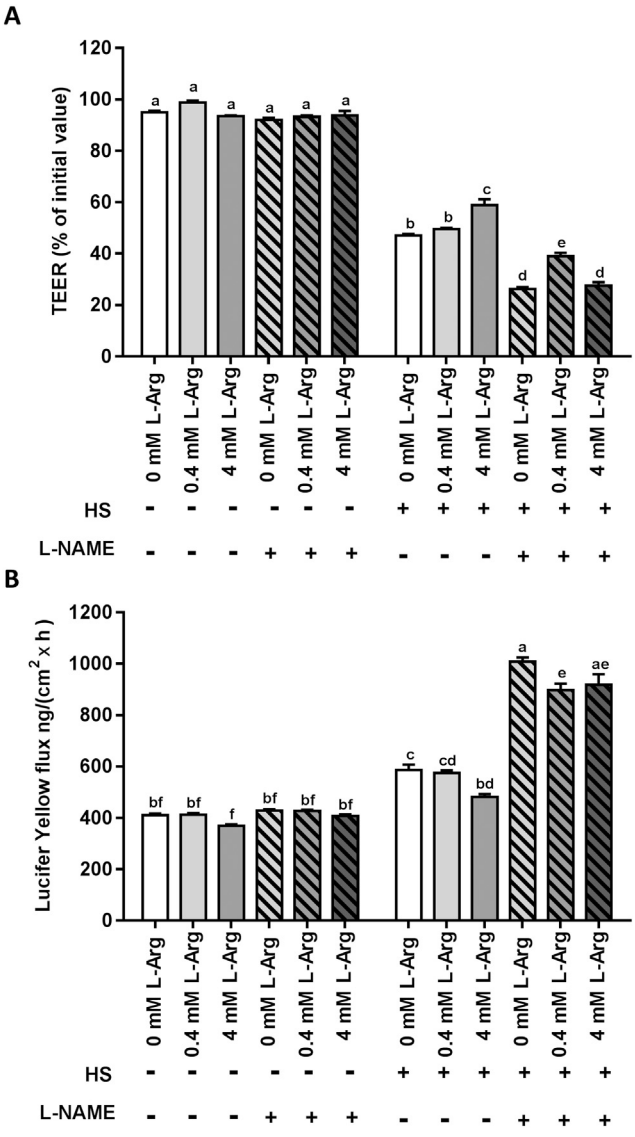

Fig. 4 – L-Arg–induced iNOS production prevents the HS-induced disruption of intestinal integrity. Caco-2 cells grown on inserts were pretreated with different concentrations of L-Arg (0, 0.4, and 4 mmol/L) in the presence or absence of L-NAME prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours to measure the TEER levels (A) or LY transport (B) across the Caco-2 monolayer. Results are expressed as a percentage of initial TEER value (A) or the amount of tracer transport [ng/(cm² × hours)] (B) as means ± SEM of 3 independent experiments each performed in triplicate. For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used. Different lowercase letters denote significant statistical differences among groups ($P < .05$).

HS conditions, pretreatment of the cells with 4 mmol/L L-Arg could prevent the HS-induced decrease of E-cadherin protein expression, and L-NAME abolished the effect of L-Arg in preserving the protein expression of E-cadherin (Fig. 5A).

Immunofluorescence staining revealed that HS-exposed cells under L-Arg deprivation (0 mmol/L) or supplemented with 0.4 mmol/L L-Arg exhibited irregular structures, suggesting clumping and internalization of fragmented E-cadherin. Pretreatment with 4 mmol/L L-Arg retained the membrane-associated localization of E-cadherin, whereas L-NAME abolished this preventive effect of L-Arg. Representative examples of the differences between cells supplemented with various L-Arg concentrations (0, 0.4, and 4 mmol/L) with or without inhibition of iNOS under control and HS conditions are depicted in Fig. 5B.

3.6. *L-Arg prevents the HS-induced decrease in iNOS protein levels, and L-NAME reverses the protective effect of L-Arg*

Western blot analysis showed that HS exposure significantly reduced the iNOS protein levels in cells cultured in medium without L-Arg. Pretreatment with 4 mmol/L L-Arg significantly prevented this HS-induced decrease of cellular iNOS levels compared to cells pretreated with 0 and 0.4 mmol/L L-Arg (Fig. 6A). In agreement with the Western blot results, visualization of the cellular localization of iNOS by immunofluorescence staining demonstrated that HS exposure under L-Arg deprivation conditions reduced the number of clearly iNOS-positive cells and that pretreatment with 4 mmol/L L-Arg modulated the expression pattern of iNOS (Fig. 6B).

Under thermal neutral conditions (37°C), no significant changes in the expression of iNOS were observed in L-Arg and/or L-NAME groups compared to the 0-mmol/L L-Arg group. Under HS conditions, inhibition of iNOS by L-NAME completely abolished the protective effect of L-Arg even at the highest concentration of 4 mmol/L L-Arg, as demonstrated by Western blot analysis and immunofluorescence staining for iNOS (Fig. 6A and B). In addition, the fluorescence intensity of the cells that expressed iNOS under HS conditions (without L-NAME) appeared to be higher compared to the cells pretreated with L-NAME (Fig. 6B). Moreover, the NO production was clearly in correspondence with the changes in iNOS protein levels, and inhibition of iNOS by L-NAME also reduced the NO production (Fig. 6C).

4. Discussion

Previously, we and others showed that HS affects gut barrier function and increases the intestinal epithelial permeability [15, 16, 27, 28]. Considering these deleterious effects of HS on the intestinal epithelium [29], we aimed to investigate the effect of L-Arg supplementation on epithelial stress injury using the human intestinal epithelial Caco-2 cells as a model.

Some limitations of this intestinal epithelial cell model need to be acknowledged. Firstly, in this in vitro model, the absorption, distribution, metabolism, and elimination of L-Arg cannot be taken into account, whereas this is rather important in the human situation. Secondly, this human colon carcinoma cell (Caco-2) model lacks some of the important characteristics of in vivo intestinal epithelium, including mucus production and interaction with luminal bacteria. There are some indications that NO donors, such as L-Arg, can promote mucus production in the intestines and reduce the bacterial translocation across gut epithelium [22, 30].

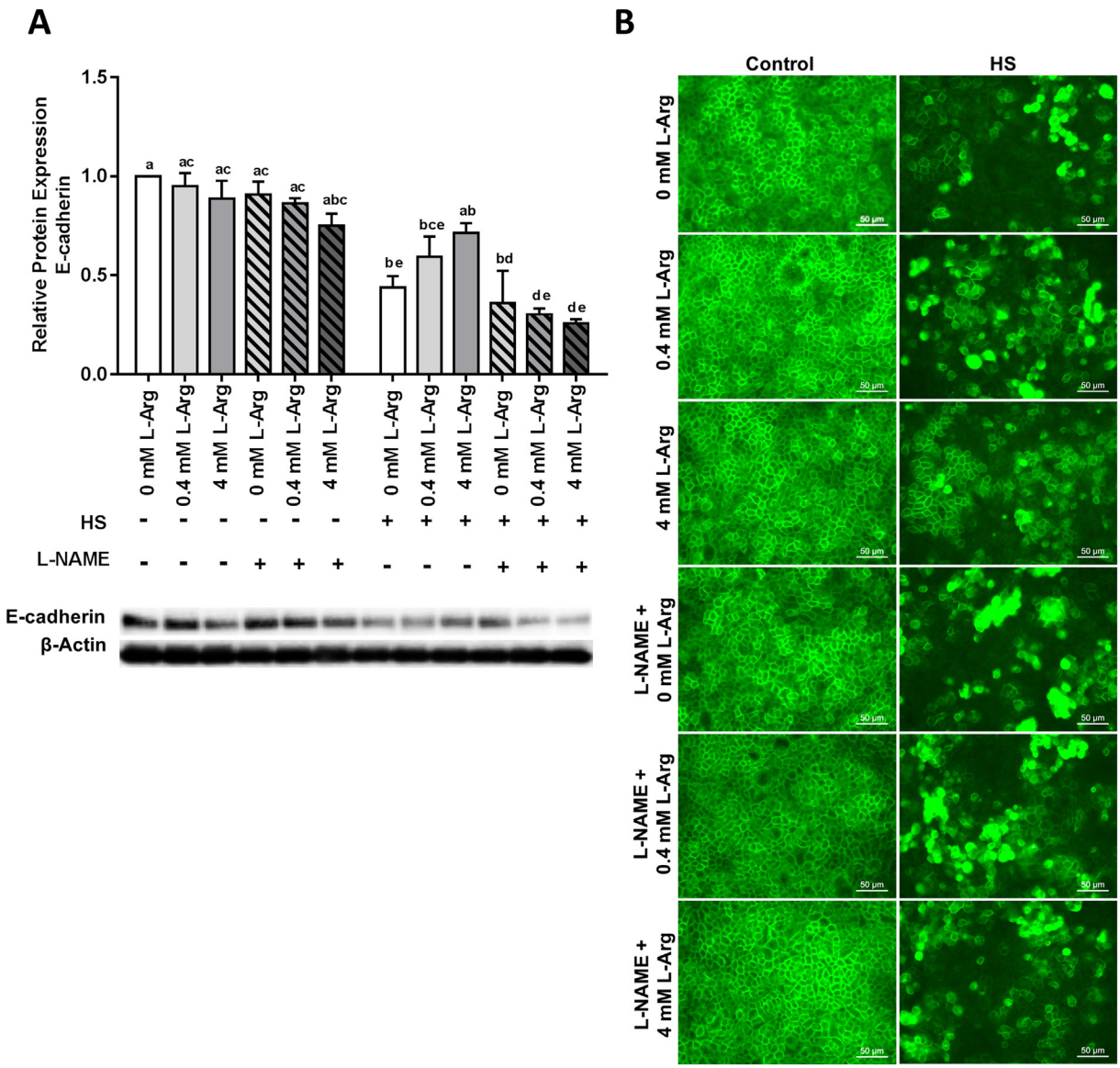

Fig. 5 – L-Arg–induced iNOS production prevents the HS-induced downregulation and delocalization of E-cadherin. Caco-2 cells grown on inserts were pretreated with different concentrations of L-Arg (0, 0.4, and 4 mmol/L) in the presence or absence of L-NAME prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours. Protein expression (A) and cellular localization (B) of E-cadherin were determined by Western blot analysis and immunofluorescence staining, respectively. Western blot results are expressed as relative protein expression, normalized to ß-actin, as means ± SEM of 3 independent experiments, and 1 representative blot has been presented (A). For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used. Different lowercase letters denote significant statistical differences among groups ($P < .05$). Representative immunofluorescence pictures (B) of the Caco-2 cells are stained with an antibody against E-cadherin (400× magnification), and scale bars represent 50 μm.

Although it has been reported that L-Arg plasma concentrations can vary between 100 and 500 μmol/L [31-33], the L-Arg concentrations in the intestinal lumen followed by postprandial luminal proteolysis can occur in the range of 1-2 mmol/L [34], highlighting the difference between plasma and intestinal concentrations. Rhoads et al showed that the maximal L-Arg effect was obtained with 4 mmol/L using an intestinal cell migration assay [34]. Chapman et al also chose 4 mmol/L L-Arg for treating neonatal piglet cells to ensure that these levels were modestly above naturally occurring levels [9]. Therefore, in the current study, the L-Arg concentrations in the range from 0.4 to 4 mmol/L were used.

The de novo synthesis of arginine in cells occurs mainly from citrulline and from aspartic acid forming the argininosuccinate, which is further converted into L-Arg and fumaric acid, a reaction catalyzed by the enzyme argininosuccinate lyase [35]. In the urea cycle, L-Arg can be converted back to L-citrulline and then be recycled back to L-Arg if required [35]. The capacity of cells to synthesize L-Arg from ornithine, citrulline, and argininosuccinate determines their

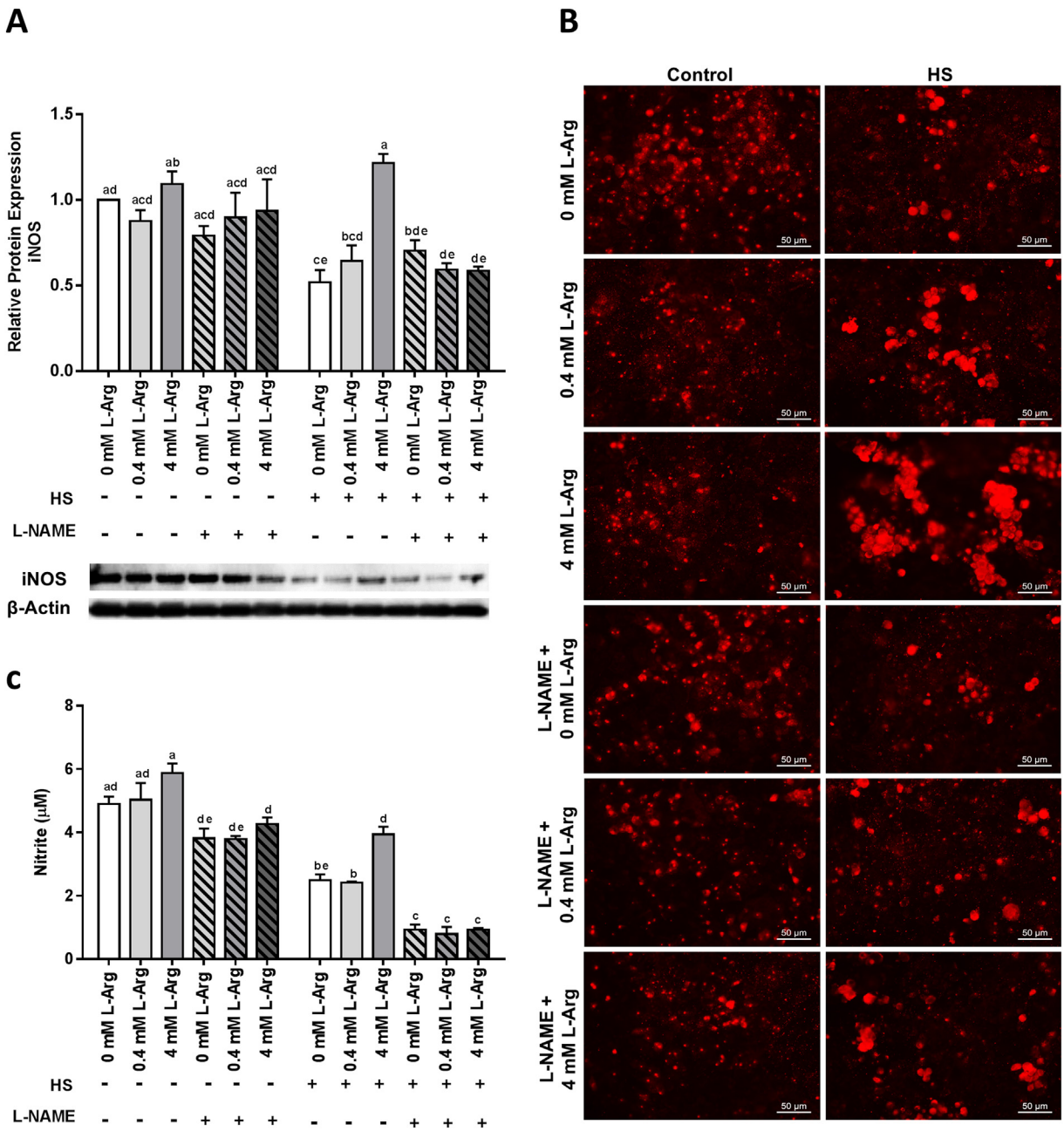

Fig. 6 – L-Arg prevents the HS-induced decrease in iNOS protein levels, and L-NAME reverses the protective effect of L-Arg. Caco-2 cells grown on inserts were pretreated with different concentrations of L-Arg (0, 0.4, and 4 mmol/L) in the presence or absence of L-NAME prior to exposure to control (37°C) or HS conditions (42°C) for 24 hours. Protein expression (A) and cellular localization (B) of iNOS was determined by Western blot analysis and immunofluorescence staining, respectively. Moreover, production of nitrite, a stable metabolite of NO, was measured with Griess reaction to evaluate the effect of iNOS inhibition on NO production (C). Results from Western blot analysis (A) are reported as means ± SEM of 3 independent experiments and are expressed as relative protein concentration normalized to β-actin (A), and 1 representative blot has been presented (A). Results from NO measurement are expressed as means ± SEM of 3 independent experiments each performed in triplicate. For comparison among the groups, 2-way ANOVA with Bonferroni post hoc test was used. Different lowercase letters denote significant statistical differences among groups ($P < .05$). Representative immunofluorescence pictures (B) of the Caco-2 cells are stained with an antibody against iNOS (400× magnification), and scale bars represent 50 µm.

vulnerability to arginine depletion, which can occur under conditions of a reduced blood flow and hypoxia, as well as other stress conditions. Moreover, cells that need to proliferate have an excessive need for L-Arg. This explains, among others, the dependency of intestinal epithelial cells on sufficient L-Arg supplies [36].

In the current study, L-Arg deprivation results in upregulated HSP70 and HO-1 mRNA expression levels, which suggest that the

endogenous cellular synthesis of L-Arg is not sufficient to meet the L-Arg needs under HS conditions. Previous in vivo investigations showed that L-Arg ameliorates the oxidative stress induction and that the deprivation of L-Arg might lead to the development of oxidative stress and ROS production [37-40].

HSP70 has an inhibitory effect on the I-κB/NF-κB pathway and subsequently on the iNOS gene expression, thereby tempering an excessive NO synthesis [41-44]. It is also known that inducible HSP70 couples to iNOS and its critical transcription factor (Kruppel-like factor 6), which in turn leads to an inhibition of iNOS gene expression [45]. Some in vivo studies have shown, however, that exposure to HS increases the production of NO under conditions of endotoxemia following HS, and this seems to represent a late inflammatory response [46, 47]. Apparently controversial responses are likely to reflect the divergence of NO effects: at moderate cellular concentrations, NO acts as a radical scavenger, whereas high concentrations result in the formation of nitrogen radicals, promoting cellular oxidative stress [5, 48-50].

L-Arg serves as a precursor of NO, and in the current study, inhibition of iNOS completely abolished the protective effect of L-Arg supplementation on the intestinal barrier dysfunction under HS conditions. The protective effect of L-Arg supplementation on intestinal integrity under stress conditions has been demonstrated in different animal models [8, 9, 19, 20, 51, 52]. A clear effect of HS on intestinal epithelial integrity was demonstrated in this study as observed by modulated mRNA and protein expression and cellular distribution of the AJ E-cadherin. As previously reported by our group, the TJ proteins are not affected by HS in this Caco-2 model [15]. NO appears to be responsible for the regulation of E-cadherin expression because inhibition of iNOS eliminated the protective effect of L-Arg. These findings are in line with studies of Vyas-Read et al who showed that L-Arg–induced NO prevents the downregulation of E-cadherin in lung epithelial cells stimulated with TGF-β1 [53]. Moreover, Nagarajan et al demonstrated that supplementation with exogenous NO protects and recovers endothelial membrane integrity by increasing the endothelial cGMP content and by rearranging actin polymerization possibly leading to regulation of AJs, including (VE)-cadherin and β-catenin [54].

The L-Arg–induced increase in NO concentrations protects cells also from lipid peroxidation, another typical sign of HS [55]. As NO is known to scavenge free radicals, the protective effect of L-Arg on barrier function and expression of AJ proteins might be also attributable to the decrease in lipid peroxidation [9]. Roig-Pérez et al demonstrated that the exaggeration of lipid peroxidation and the subsequent propagation of oxygen radicals would ultimately lead to paracellular permeability in Caco-2 cells [56].

A less commonly addressed effect of NO is its capability to attenuate the HS-induced protein tyrosine phosphorylation. Katsube et al showed that NO reduces induction of tyrosine phosphorylation of numerous cellular proteins including components of TJs and AJs; therefore, NO-mediated intracellular signal transduction might be involved in this protective function [57]. It might be possible that HS, like other oxidative stress inducers, leads to tyrosine phosphorylation of junctional complexes, resulting in the dissociation of occludin-ZO1 TJs and the E-cadherin–β-catenin AJs complexes [52, 57], ultimately leading to functional impairment of the TJ complex and loss of barrier integrity. Inhibition of tyrosine phosphorylation could therefore also contribute to the protective effects of L-Arg–induced NO.

Finally, entirely different pathways involved in the L-Arg metabolism may also contribute to its protective effect of intestinal epithelial integrity. Besides NO production, L-Arg also stimulates the ornithine synthesis via arginase enzymes [34]. Ornithine is a precursor for polyamines, which are crucial for cell growth and proliferation [36, 58]. Polyamines can regulate the expression of E-cadherin and thus play a direct a role in the maintenance of intestinal epithelial integrity [59]. Although this study did not focus on the arginase pathway, HS-exposed Caco-2 cells in medium without L-Arg showed a significantly higher arginase II mRNA expression compared to standard medium conditions (0.4 mmol/L L-Arg) (Supplemental Fig. S2). It can be suggested that this increase could be triggered by the HS-induced oxidative stress response because it is described that hypoxia increases the arginase activity as well as arginase II mRNA and protein expression via protein kinase C/RhoA/Rho kinase, mitogen-activated protein kinase, tyrosine kinases, and cyclic adenosine monophosphate/protein kinase A pathways [60, 61]. Pretreatment with 4 mmol/L L-Arg significantly increased the arginase II mRNA expression compared to the lower L-Arg concentrations in HS condition (Supplemental Fig. S2), suggesting that the polyamine production is important also for the L-Arg–induced improvement of epithelial barrier integrity.

In conclusion, the findings obtained in this study were supporting our hypothesis indicating that L-Arg supplementation protects the intestinal epithelial barrier integrity by preserving the localization and expression of E-cadherin, increasing TEER, and partially reducing the LY flux under HS. In addition, the oxidative stress response (observed by HSP70 and HO-1 expression) induced by HS was mitigated in presence of L-Arg. Whereas HS induced a significant decrease in NO production, L-Arg supplementation prevented this effect through iNOS-mediated NO synthesis. This NO synthesis is important for protecting the intestinal epithelial barrier function because by inhibiting iNOS, the effect of L-Arg on preserving TEER and E-cadherin expression and localization was abolished. The close link between a stabilized NO availability via L-Arg supplementation and the expression of E-cadherin, as part of the AJ complex responsible for the integrity of the epithelial barrier, was demonstrated for the first time in this study. Further in vivo experiments are crucial to confirm the beneficial effects of L-Arg supplementation as a strategy to enhance the resilience of animals and humans to hyperthermia-induced gastrointestinal injury.

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