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Microvascular responses to aldosterone in hamster cheek pouch microcirculation

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Abstract. The aim of the present study was to assess the *in vivo* effects of aldosterone topically applied on the hamster cheek pouch microcirculation under baseline conditions or during ischemia- reperfusion.

Male Syrian hamsters were anesthetized, tracheotomized and intubated. They were studied under baseline conditions or submitted to ischemia-reperfusion. Cheek pouch microvessels were visualized by fluorescence microscopy. Microvascular parameters were determined by computerized methods.

Under baseline conditions, aldosterone (0.2, 0.5, 2.4 μM/L/2 min) induced dose-dependent constriction of all arterioles within 2.0 ± 0.5 min of administration. Diameter reduction was in the same range in smaller arterioles: A3 ones constricted by $24 \pm 3\%$ of baseline (at the highest dose). Aldosterone applied prior to ischemia and at reperfusion caused arteriolar constriction, marked microvascular permeability (0.66 ± 0.03 Normalized Grey Level), reduction in perfused capillary ($-70 \pm 4\%$ of baseline) and leukocyte adhesion. All changes were statistically significant compared with ischemic animals.

Potassium canrenoate (mineralcorticoid receptor inhibitor) prior to aldosterone did not abolish the aldosterone-induced effects, while valsartan (angiotensin II AT_1 receptor inhibitor) prior to aldosterone ameliorated microvascular ischemia-reperfusion injury.

In conclusion, aldosterone determined dose-dependent arteriolar constriction likely by angiotensin II type-1 receptor activation (non-genomic mechanism) worsening the effects of ischemia-reperfusion on capillary perfusion, while protecting from free radical formation.

Keywords: Hamster cheek pouch microcirculation, ischemia-reperfusion, aldosterone, mineral corticoid receptor, angiotensin II AT_1 receptor

1. Introduction

Aldosterone, the steroid hormone primarily produced in the adrenal gland zona glomerulosa, is known to be effective in the regulation of renal sodium reabsorption and potassium excretion. However, recent studies indicate aldosterone is produced in cardiovascular tissues, such as heart, even the failing human heart, blood vessels and other tissues, such as the adipose one [11, 15, 26].

Aldosterone's actions are several in extra-adrenal and renal tissues. However, recently it has been assumed that aldosterone operates by rapid non-genomic mechanisms, in contrast to genomic ones inducing DNA transcription and *de novo* protein synthesis. These non-genomic effects are exploited

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within seconds or few minutes of its administration and are not blocked by spironolactone (aldosterone receptor antagonist) or inhibitors of transcription and protein synthesis, suggesting that they are independent of the mineralocorticoid receptor activation [27]. These aldosterone's non-genomic effects have been suggested to be mediated by a membrane receptor, but the involved mechanisms remain unclear.

Many findings indicate that aldosterone may exert direct vascular effects by non-genomic pathways. *In vitro* aldosterone induces vasoconstriction in afferent and efferent renal arterioles in rabbits by activation of phospholipase C with calcium mobilization through L- or T-type voltage-dependent Ca^{2+} channels [2, 3]. *In vivo* aldosterone decreases coronary blood flow in rats [8, 17], while in humans aldosterone infused into forearm causes both rapid non-genomic vasodilatory and vasoconstrictive effects [21, 22]. In vascular smooth muscle and endothelial cells, aldosterone affects the intracellular concentrations of sodium, potassium, calcium as well as the cell volume. Moreover, the Na^{+}-K^{+}-antiport is involved within minutes after aldosterone application to tissue preparations [6, 28].

An attractive issue concerns the role of aldosterone in the pathophysiology of cardiovascular injury. Clinical trials, indeed, have evidenced mineralcorticoid receptor blockade improves the survival of patients with chronic heart disease [18, 19]. These protective effects are associated with decrease in fibrosis and vascular inflammation, suggesting aldosterone is a profibrotic hormone [12]. Gros and coworkers have recently shown that acute exposure to aldosterone induces vasoconstriction through myosin light chain phosphorylation in human vascular smooth muscle cells [10]. This effect was prevented by spironolactone, suggesting that aldosterone-mediated vasoconstriction may represent an important pathophysiological mechanism in vascular disease. It is interesting to note that angiotensin II, the powerful peptide of the renin-angiotensin system, is implicated in the development of ventricular hypertrophy and cardiac fibrosis through direct or indirect activation of angiotensin II type 1 (AT_1) receptors [20, 25]. Furthermore, emerging data suggest that adipocytes may serve as a source of aldosterone, either directly or indirectly, through the release of aldosterone-stimulating factors. This finding assumes significant implications correlating aldosterone to obesity-induced increases in cardiovascular risk [5].

Today, the acute effects of aldosterone on *in vivo* peripheral microcirculation under baseline conditions or during ischemia and reperfusion have not yet been fully evaluated, although more and more data have been reported on corticosteroid hormones.

Therefore, the aim of the present study was to investigate *in vivo* effects of Aldosterone, topically administered on the hamster cheek pouch microcirculation under baseline conditions or during ischemia and reperfusion (I/R). The cheek pouch is an interesting experimental model to study the peripheral microvascular network and capillary viability by intravital fluorescence technique. To clarify the aldosterone's mechanism of action we studied the microvascular responses after inhibition of mineralcorticoid and angiotensin II AT_1 receptors by potassium canrenoate and valsartan, respectively. Our hypothesis was that aldosterone could affect arteriolar tone by promoting angiotensin II receptor AT_1 activation, as observed in other experimental models. Moreover, reactive oxygen species formation was quantified in presence of aldosterone, to assess the relationship between this steroid hormone and oxygen-derived free radical generation in the cheek pouch submitted to I/R.

2. Materials and methods

2.1. Experimental groups

Male Syrian hamsters (80–100 g) (Charles River, Calco, Italy) were used to assess the effects of aldosterone on cheek pouch microcirculation under baseline conditions. We utilized 15 animals divided

in five experimental groups: D_1, D_2 and D_3 ($n = 3$ for each group) were treated with topical aldosterone 0.2, 0.5 and 2.4 μM/L/2 min, respectively. Group P ($n = 3$) was treated with topical potassium canrenoate 1 mM/L/2 min and group V ($n = 3$) was treated with topical valsartan 0.4 mM/L/2 min. Moreover, we submitted the following groups of hamsters to cheek pouch ischemia and reperfusion injury. The first group (group I: $n = 13$) was treated with topically applied vehicle and subjected to 30 min of ischemia and 30 min of reperfusion. The second group (subgroups: A_1, A_2 and A_3, $n = 13$ for each subgroup) was treated with topically applied aldosterone 0.2, 0.5 and 2.4 μM/L/2 min, respectively, starting 10 min before ischemia and at the beginning of reperfusion. The third group of animals (group PA_3, $n = 13$) was exposed to topically administered potassium canrenoate (1 mM/L/2 min) starting 10 min prior to aldosterone before ischemia and at the beginning of reperfusion (2.4 μM/L/2 min); the fourth group (group VA_3, $n = 13$) was treated with topically applied valsartan (0.4 mM/L/2 min) starting 10 min prior to aldosterone before ischemia and at the beginning of reperfusion (2.4 μM/L/2 min). The last group of animals (group S or sham-operated, $n = 8$) was submitted to the same surgical procedures without ischemia and reperfusion injury.

In pilot studies, we used different dosages of potassium canrenoate (0.1–3 mM/L/2 min) or valsartan (0.1–2 mM/L/2 min) to choose the appropriate dose for our experimental groups. We chose intermediate dosages for the present study, according to previous data [23, 24].

In each experimental group seven hamsters (five in sham-operated group) were used for *in vivo* microvascular studies, and six animals (three in sham-operated group) were utilized for quantitative determination of oxidative stress by evaluation of thiobarbituric acid-reactive substances (TBARS).

All experiments conform to the Guide for the Care an Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to institutional rules for the care and handling of experimental animals.

2.2. Animal preparation

The cheek pouch was surgically prepared as previously reported [4]. Hamsters were anesthetized (pentobarbital sodium, Nembutal, 5 mg/100 g body weight i.p.) and tracheotomized. The right femoral artery and femoral vein were cannulated to measure blood pressure and administer additional anesthesia and fluorescent tracers. The cheek pouch was gently everted and fixed to a special modified stage of the microscope; a thin black blade was inserted through a small incision between the upper and lower layers of the pouch, thus preserving the epithelial cells. The membrane was suffused with Ringer solution (in mM: 137 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.0 $CaCl_2$, 18.0 $NaHCO_3$) at 36.0 ± 0.5°C bubbled with 5% CO_2-95% N_2 adjusted to pH 7.35.

Atraumatic microvascular clips were placed for 30 min on the proximal part of the cheek pouch to achieve complete ischemia. After removing clamp, the microcirculation was observed for 30 min (reperfusion).

2.3. Fluorescence microscopy technique

Observations of microcirculation were made with a fluorescent microscope (Leitz Orthoplan) with objectives 4x, numerical aperture (NA) 0.14; 20x, NA 0.25; 32x, NA 0.40, a 10x eyepiece and a filter block (Ploemopak, Leitz). Epi-illumination was provided by a 100 W mercury lamp using the appropriate filters for fluorescein isothiocyanate (FITC) bound to dextran (molecular weight 150 kDa, 50 mg/100 g body weight i.v. as 5% wt/vol solution in 5 min) (FD 150) (Sigma Chemical), for rhodamine 6G and a

heat filter (Leitz KG1). The animals received an intravenous injection of 1 mg rhodamine 6G/100 g body weight in 0.3 ml (Sigma Chemical) and a supplemental injection (final volume $0.3\,\text{ml} \cdot 100\,\text{g}^{-1} \cdot \text{h}^{-1}$) throughout the experiment to label leukocytes for adhesion evaluation.

The area of interest was televised with a DAGE MTI 300 low-light level digital camera and observed from a Sony PVM 122 CE monitor. Video images were videotaped and microvascular measurements were made off-line using a computer assisted imaging software system (MIP Image, CNR, Institute of Clinical Physiology, Pisa, Italy).

Microvascular observations were carried out for 1 min every 5 min during application of drugs; then, every 10 min during I/R.

2.4. Microvascular parameters evaluation

In each preparation the arteriolar vessels were classified according to a centrifugal ordering scheme from the largest A1 (mean diameter: $50.0 \pm 3.5\,\mu$m) to smaller A2 (mean diameter: $28.5 \pm 4.0\,\mu$m) and A3 (mean diameter $14.5 \pm 0.6\,\mu$m) up to the smallest A4 (mean diameter: $7.8 \pm 0.7\,\mu$m) arterioles. We tried to investigate the responses of each arteriolar order to the experimental conditions. However, we chose to present only the data regarding A3 arterioles, because the responses were in the same range in smaller arterioles (A3-A4), but the size of order 3 arterioles was more appropriate for evaluation of diameter changes.

The increase in permeability was manifested by extravasation of FITC-dextran, which appeared as fluorescent spots outside microvessels. The increase in permeability was calculated and reported as Normalized Grey Levels (NGL): NGL = (I – Ir)/Ir, where Ir is the average baseline grey level at the end of vessel filling with fluorescence (average of 5 windows located outside the blood vessels with the same windows being used throughout the experimental procedure), and I is the same parameter after ischemia or reperfusion. Grey levels ranging from 0 to 255 were determined by the MIP Image program in windows measuring $50 \times 50\,\mu$m.

The perfused capillary length (PCL) was measured by MIP Image in an area of $550 \times 550\,\mu$m. In this system, the length of perfused capillaries is easily established by the automated process because it is outlined by dextran.

Adherent leukocytes highlighted with rhodamine 6G (i.e. cells on vessel walls that did not move over a 30 second observation period) were quantified in terms of numbers/100 μm venular length/30 s.

Vessel diameters and capillary red blood cell velocity (V_{RBC}) were measured with a computer-assisted method (frame by frame). The results of diameter measurements were in accord with those obtained by shearing method ($\pm 0.5\,\mu$m). To avoid bias due to single operator measurements, two independent “blinded” operators determined all the measured parameters. Their measurements overlapped in all cases.

Mean arterial blood pressure (Viggo-Spectramed P10E2 transducer – Oxnard, CA – connected to a catheter in the carotid artery) and heart rate were monitored with a Gould Windograf recorder (model 13-6615-10 S, Gould, OH, USA). Data were recorded and stored in a computer.

2.5. Lipid peroxidation quantification

Malondialdehyde (MDA), a measure of lipoperoxidation, was assayed in the form of tissue content of thiobarbituric acid reactive substances, according to the method previously reported [9, 16]. Briefly, 0.25 g of cheek pouch membrane were homogenized in 5% tricloroacetic acid, in presence of butilated hydroxytoluene (0.01%) and 1 mM EDTA to avoid lipid peroxidation during assay. The precipitate was

pelleted by centrifugation (3000 rpm for 15 min) and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) thiobarbituric acid. Subsequently, the samples were heated at 80°C for 30 min and after cooling the absorption at 532 nm was measured.

TBARS were expressed as MDA amount using freshly diluted MDA bisdimethylacetal as standard. TBARS in check pouch membrane were evaluated at different times: at the end of ischemia ($n = 3$) and at 20 min of reperfusion ($n = 3$).

2.6. Statistical analysis

All presented values are means ± SEM. Data were tested for normal distribution with the Kolmogorov-Smirnov test. Parametric (Student's tests, Anova and Bonferroni post hoc test) or nonparametric tests (Wilcoxon, Mann-Whitney and Kruskal-Wallis tests) were used; nonparametric tests were applied to compare diameter and length data among experimental groups. The statistical analysis was carried out by SPSS 14.0 statistical package. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Arteriolar responses to Aldosterone

Topically applied aldosterone under basal conditions (groups D_1, D_2 and D_3) induced a dose-dependent significant diameter reduction in all arteriolar orders. These changes were observed within 2.0 ± 0.5 min of its administration and lasted 3–10 min according to dosage. Highest dosage aldosterone constricted A3 arteriole diameter by 24 ± 3% of baseline ($p < 0.01$ vs. baseline) (Fig. 1 and Tables 1, 2). Vasoconstriction was accompanied by V_{RBC} increase (0.28 ± 0.20 mm/s, $p < 0.01$ vs. baseline), while there was no leukocyte adhesion. Vascular permeability and PCL did not show significant changes compared with baseline.

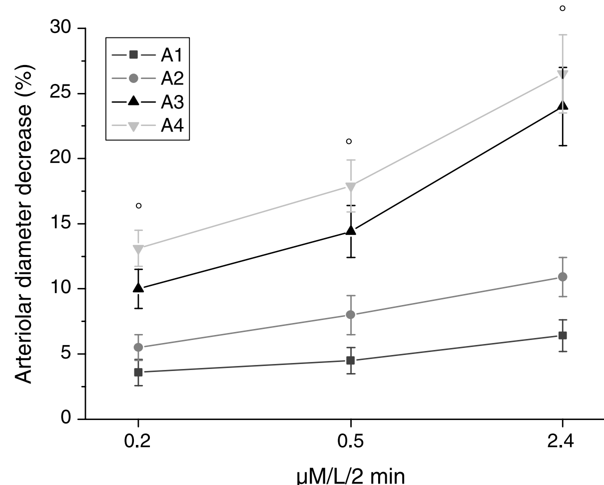

Fig. 1. Percent decrease in arteriolar diameter induced by topically applied aldosterone at the dosages of 0.2, 0.5 and 2.4 μM/L/2 min (D_1, D_2 and D_3 group, respectively). °$p < 0.01$ vs. S group.

Table 1

Drug treatment, doses and route of administration in the different experimental groups. (I/R: animals subjected to ischemia and reperfusion and NO I/R: animals not subjected to ischemia and reperfusion, N: number of hamsters utilized)

Groups	*N*	I/R plus treatement	Time	Groups	*N*	NO I/R plus treatement	Time
I	13	Saline solution topically applied	Prior to ischemia At reperfusion beginning	S	8	Saline solution topically applied	Twice at 40 min interval
A_1	13	Aldosterone 0.2 μM/L/2 min topically applied	Prior to ischemia At reperfusion beginning	D_1	3	Aldosterone 0.2 μM/L/2 min topically applied	Once
A_2	13	Aldosterone 0.5 μM/L/2 min topically applied	Prior to ischemia At reperfusion beginning	D_2	3	Aldosterone 0.5 μM/L/2 min topically applied	Once
A_3	13	Aldosterone 2.4 μM/L/2 min topically applied	Prior to ischemia At reperfusion beginning	D_3	3	Aldosterone 2.4 μM/L/2 min topically applied	Once
PA_3	13	Potassium canreonate 1 mM/L/2 min topically applied	Prior to Aldosterone	P	3	Potassium canreonate 1 mM/L/2 min topically applied	Once
		Aldosterone 2.4 μM/L/2 min topically applied	Prior to ischemia At reperfusion beginning	V	3	Valsartan 0.4 mM/L/2 min topically applied	Once
VA_3	13	Valsartan 0.4 mM/L/2 min topically applied	Prior to Aldosterone				
		Aldosterone 2.4 μM/L/2 min topically applied	Prior to ischemia At reperfusion beginning				

Under baseline conditions, the topical application of potassium canrenoate (1 mM/L/2 min) or valsartan (0.4 mM/L/2 min) did not affect arteriolar diameter and the other microvascular parameters (group P and V, respectively) (Table 2).

3.2. Arteriolar responses to ischemia and reperfusion

3.2.1. Sham-operated animals

In the sham-operated animals (group S) there were no changes in arteriolar diameter nor increase in permeability nor leukocyte adhesion during baseline observations. All capillaries were perfused and V_{RBC} was 0.21 ± 0.02 mm/s (Tables 1, 3).

3.2.2. Ischemic hamsters

In the ischemic hamsters, occlusion of vessels supplying the cheek pouch for 30 min caused blood flow to stop and marked vascular permeability; at the end of ischemia NGL were 0.31 ± 0.06 ($p < 0.01$ vs. baseline: 0.03 ± 0.02 NGL). At the end of reperfusion, A3 arteriole diameter decreased by $19 \pm 3\%$ of baseline ($p < 0.01$ vs. baseline and group S), macromolecular leakage increased (0.58 ± 0.04 NGL,

Table 2

Variations of the main parameters in hamsters treated with highest dose aldosterone, potassium canrenoate and valsartan under baseline conditions (D_3, P and V group, respectively). Changes of arteriolar diameter is reported as percent changes of 100% baseline values (baseline diameter of order 3 arterioles = 100). Capillary red blood cell velocity (V_{RBC}) is expressed in mm/s

Group	Arteriolar diameter (%)	Capillary red blood cell velocity (V_{RBC}) (mm/s)
Baseline	100 ± 2	0.21 ± 0.02
D_3	$76 \pm 3^{\wedge}$	$0.28 \pm 0.20^{\wedge}$
P	$101.0 \pm 0.5^{\S}$	$0.21 \pm 0.03^{\S}$
V	$101.0 \pm 0.5^{\S}$	$0.22 \pm 0.01^{\S}$

$^{\wedge}p < 0.01$ vs. S group, $^{\S}p < 0.01$ vs. D_3 group.

Table 3

Variations of the main parameters in sham-operated group (S), ischemic group (I), ischemic hamsters treated with high dose aldosterone (A_3), ischemic hamsters treated with potassium canrenoate plus high dose aldosterone (PA_3), ischemic hamsters treated with valsartan plus high dose aldosterone (VA_3). Variations of arteriolar diameter and capillary perfusion are reported as percent changes of 100% baseline values (baseline diameter of order 3 arterioles = 100 and perfused capillary length under baseline conditions = 100). NGL: Normalized Grey Levels. Leukocyte adhesion is reported as number of leukocytes/100 μm of venular length/30 seconds and capillary red blood cell velocity (V_{RBC}) is expressed in mm/s. EA: prior to ischemia, EI: at the end of ischemia, ER: at the end of reperfusion

Group	Arteriolar diameter (%)	Microvascular permeability (NGL)	Leukocyte adhesion (Number of leukocyte/100 μm of venular length/30 s)	Perfused capillary length (PCL) (%)	Capillary red blood cell velocity (V_{RBC}) (mm/s)
S	100 ± 3	0.02 ± 0.01	1.0 ± 0.5	100 ± 5	0.21 ± 0.02
I	$81 \pm 3^{\circ}$	0.31 ± 0.06 EI$^{\circ}$	$11 \pm 2^{\circ}$	$40 \pm 4^{\circ}$	0.20 ± 0.02
		0.58 ± 0.04 ER$^{\circ}$			
A_3	90 ± 2 EA$^{\circ *}$	0.42 ± 0.04 EI$^{\circ}$	$4 \pm 2^{\circ *}$	$30 \pm 3^{\circ *}$	$0.28 \pm 0.02^{\circ *}$
	73 ± 3 ER$^{\circ *}$	0.66 ± 0.03 ER$^{\circ}$			
PA_3	91 ± 4 EA$^{\circ *}$	0.40 ± 0.02 EI$^{\circ}$	$5 \pm 2^{\circ *}$	$32 \pm 3^{\circ *}$	$0.27 \pm 0.02^{\circ *}$
	74 ± 2 ER$^{\circ *}$	0.65 ± 0.03 ER$^{\circ}$			
VA_3	110.5 ± 3.2 ER$^{*\#}$	0.22 ± 0.03 EI$^{\circ *\#}$	$4 \pm 2^{\circ *}$	$65 \pm 2^{\circ *\#}$	$0.19 \pm 0.03^{\#}$
		0.35 ± 0.02 ER$^{\circ *\#}$			

$^{\circ}p < 0.01$ vs. S group, $^{*}p < 0.01$ vs. I group, $^{\#}p < 0.01$ vs. A_3 subgroup.

$p < 0.01$ vs. baseline) and leukocyte adhesion was pronounced compared with baseline (11 ± 2/100 μm of venular length/ 30 s, $p < 0.01$ vs. baseline) in V1 venules (mean diameter: 16.8 ± 3.9 μm), (Figs. 2A, B, 3, 4). PCL was reduced by $60 \pm 4\%$, $p < 0.01$ compared with baseline; V_{RBC} (0.20 ± 0.02 mm/s) did not change at the end of reperfusion when compared with baseline (Fig. 5, Tables 1, 3).

3.2.3. Aldosterone plus I/R

Animals treated with the highest dose of aldosterone (subgroup A_3), topically applied before ischemia, showed a decrease in A3 arteriole diameter by $10 \pm 2\%$ of baseline within 2.0 ± 0.5 min of its

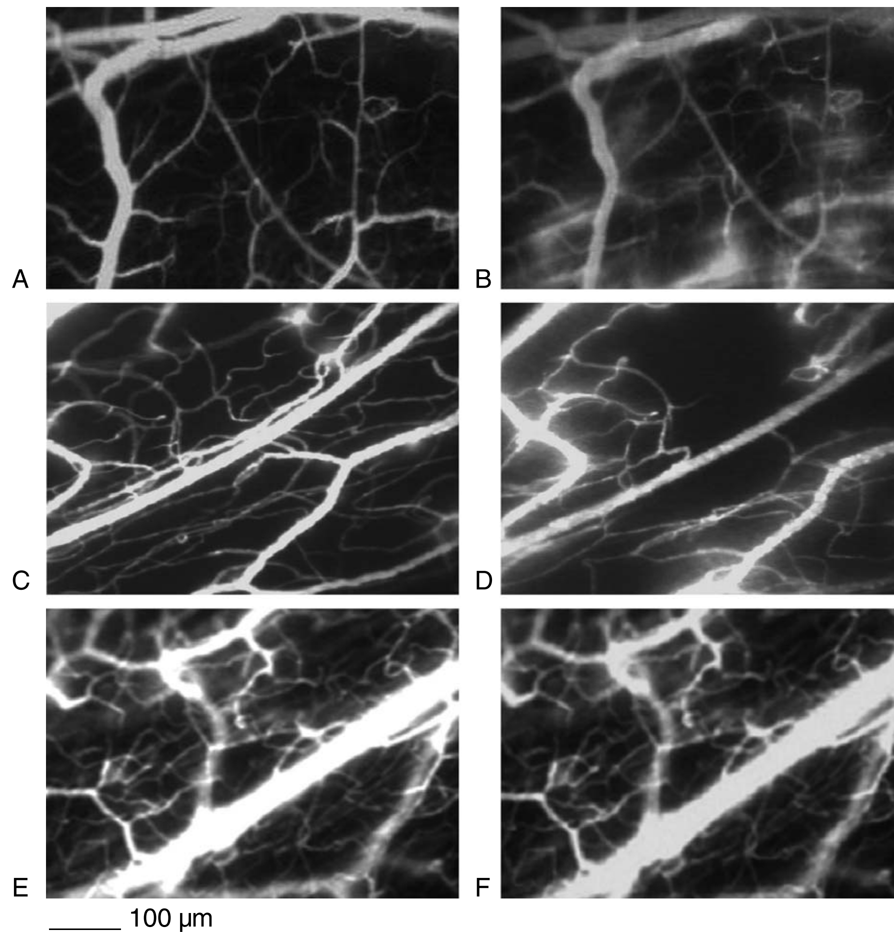

Fig. 2. Computer-assisted image of cheek pouch microvessels under baseline conditions (A) and at the end of reperfusion (B) in an ischemic hamster. The increase in permeability is outlined by the marked change in the colour of interstitium (from black to white). Computer-assisted image of cheek pouch microvessels under baseline conditions (C) and at the end of reperfusion (D) in an aldosterone-treated hamster (2.4 μM/L/2 min). Computer-assisted image of cheek pouch microvessels under baseline conditions (E) and at the end of reperfusion (F) in a valsartan (0.4 mM/L/2 min) plus aldosterone - treated hamster (2.4 μM/L/2 min). In the last hamster there was no leakage of fluorescent dextran. Scale bar = ___ 100 μm.

administration. At the end of ischemia, fluorescent dextran leakage significantly increased compared with ischemic group (0.42 ± 0.04 NGL, $p < 0.01$ vs. ischemic group) (Fig. 3).

At the end of reperfusion, A3 arteriole diameter was reduced by 27 ± 3% of baseline ($p < 0.01$ vs. ischemic group); vascular permeability was pronounced along the venular side of microcirculation (0.66 ± 0.03 NGL, $p < 0.01$ vs. ischemic group) (Figs. 2 C, D, 3). The number of adherent leukocytes significantly decreased compared with ischemic animals (4 ± 2/100 μm of venular length/30 s, $p < 0.01$ vs. group S and ischemic), PCL diminished by 70 ± 3% of baseline ($p < 0.01$ vs. groups S and I), while V_{RBC} increased to 0.28 ± 0.02 mm/s ($p < 0.01$ vs. groups S and I) (Figs. 4, 5, Tables 1, 3). It is worth noting that the decrease in capillary perfusion was accompanied by venular remodeling with reduction of viable vessels.

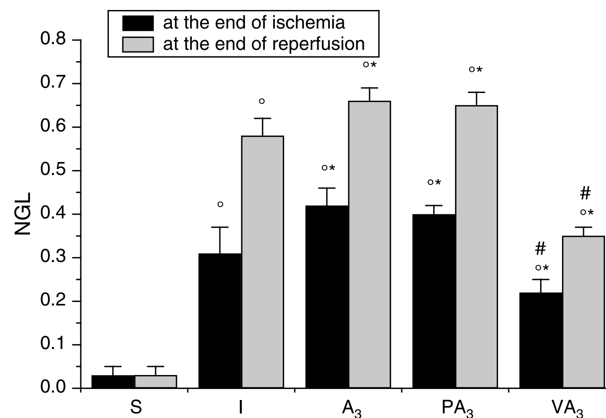

Fig. 3. Permeability increase, expressed as Normalized Grey Levels, NGL, after 30 min of ischemia (left) and at the end of reperfusion (right) in the experimental groups: S = sham-operated group ($n=5$); I = ischemic group ($n=7$); A_3 = aldosterone 2.4 μM/L/2 min topically applied, subgroup ($n=7$), PA_3 = potassium canrenoate (1 mM/l/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n=7$), VA_3 = valsartan (0.4 mM/L/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n=7$). $^{\circ}p<0.01$ vs. S group, $^{*}p<0.01$ vs. I group, $^{\#}p<0.01$ vs. A_3 subgroup.

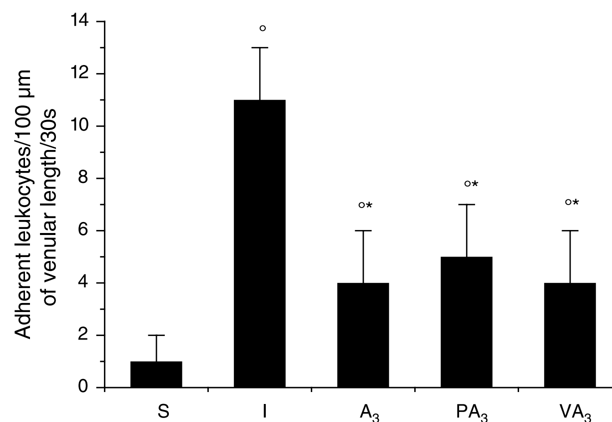

Fig. 4. Leukocyte adhesion expressed as number of adherent leukocytes/100 μm of venular length/30 s at the end of reperfusion in the experimental groups: S = sham-operated group ($n=5$); I = ischemic group ($n=7$); A_3 = aldosterone 2.4 μM/L/2 min topically applied, subgroup ($n=7$), PA_3 = potassium canrenoate (1 mM/l/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n=7$), VA_3 = valsartan (0.4 mM/L/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n=7$). $^{\circ}p<0.01$ vs. S group, $^{*}p<0.01$ vs. I group.

3.2.4. Potassium canrenoate plus I/R

Potassium canrenoate locally administered prior to aldosterone (group PA_3) did not prevent the aldosterone-induced A3 arteriole diameter decrease, while microvascular permeability increased (NGL: 0.40 ± 0.02, $p<0.01$ vs. ischemic group) at the end of ischemia (Fig. 3). At the end of reperfusion, all arterioles constricted as observed in hamsters treated with highest dosage aldosterone. Microvascular leakage was marked and NGL were 0.65 ± 0.03 ($p<0.01$ vs. ischemic group) (Fig. 3). The number of adherent leukocytes was 5 ± 2/100 μm of venular length/ 30 s ($p<0.01$ vs. ischemic group) and PCL was

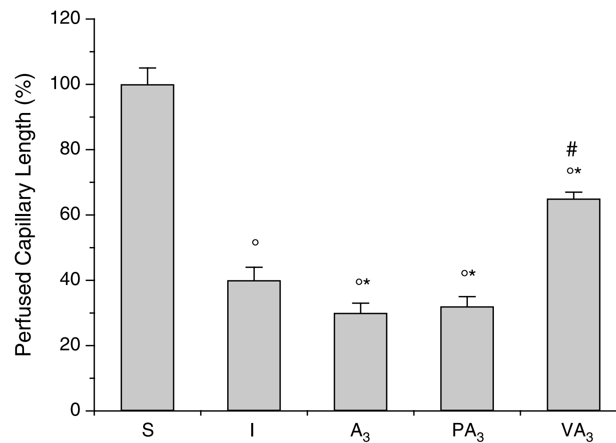

Fig. 5. Perfused capillary length (PLC) at the end of reperfusion in the experimental groups: S = sham-operated group ($n = 5$); I = ischemic group ($n = 7$); A_3 = aldosterone 2.4 μM/L/2 min topically applied, subgroup ($n = 7$), PA_3 = potassium canrenoate (1 mM/l/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n = 7$), VA_3 = valsartan (0.4 mM/L/2 min) plus aldosterone (2.4 μM/L/2 min) topically applied, group ($n = 7$). $^{\circ}p < 0.01$ vs. S group, $^{*}p < 0.01$ vs. I group, $^{\#}p < 0.01$ vs. A_3 subgroup.

reduced by 68 ± 3%, ($p < 0.01$ vs. ischemic group) (Figs. 4, 5, Tables 1, 3). V_{RBC} was as high as observed in subgroup A_3 (0.27 ± 0.02 mm/s, $p < 0.01$ vs. ischemic group).

3.2.5. Valsartan plus I/R

Valsartan locally applied prior to aldosterone (group VA_3) abolished A3 arteriole diameter reduction observed at the end of ischemia, while microvascular permeability was partially prevented (0.22 ± 0.03 NGL, $p < 0.01$ vs. ischemic and A_3 groups) at the end of ischemia (Fig. 3).

At the end of reperfusion all arteriolar diameters increased (A3: by 10.5 ± 3.2% of baseline, $p < 0.01$ vs. groups S, I, A_3 and PA_3); vascular leakage was attenuated compared with subgroup A_3 (0.35 ± 0.02 NGL, $p < 0.01$ vs. groups I and A_3) (Figs. 2E, F, 3). Leukocyte adhesion was reduced (4 ± 2/100 μm of venular length/30 s, $p < 0.01$ vs. ischemic group) while PCL was partially protected (decrease by 35 ± 2% of baseline, $p < 0.01$ vs. groups S, I, PA_3 and A_3) and V_{RBC} was 0.19 ± 0.03 mm/s ($p < 0.01$ vs. subgroup A_3) (Figs. 4, 5, Tables 1, 3).

Aldosterone, potassium canrenoate and valsartan did not modify cardiovascular parameters, such as mean arterial blood pressure and heart rate, unchanged in the experimental groups.

4. TBARS

In each experimental group TBARS were evaluated at the end of ischemia and at 20 min of reperfusion on the cheek pouch homogenate. In sham-operated animals there were no significant amounts of TBARS at the end of observation. In ischemic hamsters, at the end of ischemia TBARS increased by 62 ± 4% of baseline MDA, as quantified in contralateral cheek pouch, while at 20 min of reperfusion they increased by 64.2 ± 3.7% of baseline MDA. Aldosterone topically applied prior to ischemia significantly reduced TBARS concentration in dose-dependent manner: at the end of ischemia and at 20 min of reperfusion TBARS did not significantly change compared with contralateral cheek pouch MDA at the highest dosage: +2.1 ± 0.8% MDA and +2.0 ± 0.7% MDA, respectively, $p < 0.01$ vs. ischemic hamsters.

Potassium canrenoate or valsartan prior to high dosage aldosterone did not abolish aldosterone's effects.

5. Discussion

The results of the present study indicate that the topical administration of aldosterone caused immediate reduction of arteriolar diameter in hamster cheek microcirculation under baseline conditions, indicating that these effects may be triggered by aldosterone-activated non-genomic mechanisms. Moreover, we noted aldosterone induced higher constriction in smaller arterioles (A3, A4); this pattern of responses has been observed in previous studies showing the terminal and precapillary arterioles more susceptible to vasoactive substances, such as norepinephrine, for their functional and structural characteristics [1, 7].

Moreover, our data demonstrate ischemia and the subsequent reperfusion of the hamster cheek pouch caused significant permeability increase, marked leukocyte adhesion accompanied by reduced capillary perfusion. The microvascular responses were characterized by significant changes in arteriolar diameters decreasing at the end of ischemia and reperfusion.

Aldosterone local administration prior to ischemia increased microvascular damage compared with ischemic hamsters. At the end of reperfusion arterioles significantly constricted, vascular leakage increased, localized around connecting and postcapillary venules, while leukocyte adhesion was reduced. It is worth underlying vasoconstriction induced a significant reduction in capillary perfusion.

These microvascular alterations, such as the decrease in diameter, occurred in few minutes; therefore, it is possible to hypothesize that aldosterone's mechanism of action on peripheral microcirculation might be non-genomic. To investigate aldosterone intracellular signaling pathways we administered potassium canrenoate, an intracellular mineralcorticoid receptor (MR) inhibitor [21]. The results indicate that topically applied potassium canrenoate prior to aldosterone did not significantly affect aldosterone-induced effects.

We were interested to the effects of valsartan, an angiotensin II AT_1 receptor antagonist, because previous data indicate valsartan is able to interfere with aldosterone effects on coronary arteriolar constriction [13]. In particular, valsartan abolished aldosterone-induced constriction in isolated coronary arterioles. In our model valsartan prior to aldosterone prevented arteriolar diameter decrease and vascular permeability increase at the end of both ischemia and reperfusion. At the end of reperfusion leukocyte adhesion was reduced, while capillary perfusion was preserved.

Therefore, aldosterone-induced vasoconstriction during ischemia and reperfusion may be triggered by activation or facilitation of angiotensin II type-1 receptors, while the blockade of MR receptor is ineffective in contrasting aldosterone's effects. Our data support the results of the previous study carried out on coronary arterioles of normal and hypertensive rats, indicating that aldosterone-induced constriction of coronary arterioles is enhanced by genetically defined hypertension [13]. Aldosterone is effective in increasing the angiotensin II AT_1 receptor density, facilitating the myocardial fibrosis induced by cardiac angiotensin II [18]. However, these effects may be due to genomic mechanism triggered by aldosterone. In our model the effects on angiotensin II AT_1 receptors can be rapidly observed, because valsartan was able to abolish arteriolar constriction at the beginning of ischemia and to protect cheek pouch microvasculature from ischemia and reperfusion injury. These protective effects were not observed after MR inhibition by potassium canreonate.

During ischemia and reperfusion aldosterone was able to prevent hamster cheek pouch membrane lipid peroxidation, because all treated animals showed a reduced TBARS concentration. Therefore, damage

due to aldosterone appears unrelated to membrane lipoperoxidation or increase in oxygen-derived free radical formation. Microvascular impairment was mainly due to constriction of arteriolar smooth muscle cells and consequent dramatic reduction in capillary perfusion and venular drainage. These changes were accompanied by marked venular remodeling due to no reflow phenomenon on the venular side of microcirculation. The interesting issue was that aldosterone induced arteriolar constriction corresponded to reduction in free radical formation and decrease in leukocyte adhesion. These events, however, were not able to counteract the decrease in arteriolar diameter and in tissue perfusion. Therefore, it is likely to hypothesize that facilitation of angiotensin II effect on the vessel wall could trigger vasoconstriction and consequent changes in blood hemorheological properties and activation of intravessel clotting cascade. However, further studies are required to clarify these interesting interaction of steroid hormones and angiotensin II on vessel wall cell populations. The effects of steroids on vascular wall cells are well known as permissive ones, such as the permissive effect of cortisol on arteriolar tone [14]. Aldosterone effect might be permissive on angiotensin II receptor, facilitating a supramaximal constriction as the final result.

In conclusion, aldosterone caused vasoconstriction by angiotensin II type-1 receptor activation or facilitation, worsening the effects of ischemia and reperfusion on capillary perfusion, while protection from free radical formation was less effective. The net results of these conflicting mechanisms were the dramatic reduction in capillary network perfusion and decrease in blood flow supply to tissues.

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