Effect of Angiotensin II and Losartan on the Phagocytic Activity of Peritoneal Macrophages from Balb/C Mice

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Angiotensin II (AII), a product of rennin-angiotensin system, exerts an important role on the function of immune system cells. In this study, the effect of AII on the phagocytic activity of mouse peritoneal macrophages was assessed. Mice peritoneal macrophages were cultured for 48 h and the influence of different concentrations of AII (10^{-14} to 10^{-7} M) and/or losartan, 10^{-16} to 10^{-6} M), an AT_1 angiotensin receptor antagonist, on phagocytic activity and superoxide anion production was determined. Dimethylthiazoldiphenyltetrazolium bromide reduction and the nucleic acid content were used to assess the cytotoxicity of losartan. A stimulatory effect on phagocytic activity ($P < 0.05$) was observed with 10^{-13} M and 10^{-12} M AII concentrations. The addition of losartan (up to10^{-14} M) to the cell cultures blocked ($P < 0.001$) the phagocytosis indicating the involvement of AT_1 receptors. In contrast, superoxide anion production was not affected by AII or losartan. The existence of AT_1 and AT_2 receptors in peritoneal macrophages was demonstrated by immunofluorescence microscopy. These results support the hypothesis that AII receptors can modulate murine macrophage activity and phagocytosis, and suggest that AII may have a therapeutic role as an immunomodulatory agent in modifying the host resistance to infection.

Key words: angiotensin receptor - cytotoxicity peritoneal macrophage - phagocytosis

In recent years, much insight has been gained in the biochemical pathways by which angiotensin II (AII) exerts its effects on cells. In particular, the cloning of the A II receptors AT_{1A}, AT_{1B}, and AT_2 has led to better understanding of how AII interacts with different cells to produce its physiological responses (Regitz-Zagrosek et al. 1996, Lavoie & Sigmund 2003).

The renin-angiotensin system (RAS) is an important regulatory mechanism for cardiovascular homeostasis in normal and hypertensive subjects (Brewster et al. 2003, Lavoie & Sigmund 2003). Previous reports have shown that macrophages from different tissues express components of the RAS (Nickening et al. 1997, Lamparter et al. 1998) and granuloma macrophages can synthesize angiotensin (Weinstock & Blum 1987). In addition, high levels of renin mRNA are expressed by monocytes and macrophages in the necrotic myocardium (Iwai et al. 1996). The ability of AII to stimulate cardiac fibroblast proliferation and extracellular matrix (ECM) synthesis by these cells suggests an important role for AII in ventricular remodeling. This conclusion agrees with the demonstration that AII can influence the accumulation of ECM and the synthesis of type I collagen, fibronectin, biglycan, and growth factors (Wolf et al. 1992, Matisusaka & Ichikawa 1997).

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The presence of specific AII binding sites in macrophages has been suggested by several studies (Dezso & Fóris 1981, Thomas & Hoffman 1984, Dezso et al. 1988). Prabha et al. (1990) were the first to show that at physiological concentrations, AII can increase free-radical generation by polymorphonuclear neutrophils. Kumar and Das (1993) subsequently confirmed this observation. Hydrogen peroxide, peroxide, and superoxide production via AT_1 receptors is an important component in the respiratory burst response that can be used to measure the activation of macrophages (Scheidegger et al. 1997, Jaimes et al. 1998, Pueyo et al. 1998, Yanagitani et al. 1999).

Based on these reports, this study was designed to investigate the role of AT_1 receptors in the phagocytic activity and superoxide release of mouse peritoneal macrophages. The effect of AII on these cells was determined in the absence and presence of losartan, an AT_1 receptor antagonist. We also evaluated the cytotoxic effect of losartan and the presence of AT_1 and AT_2 receptors on peritoneal macrophages.

MATERIALS AND METHODS

Cultures of macrophages - Male Balb/C mice 6-8 weeks old were supplied by the Central Animal House (Unicamp). The experimental protocols described here were approved by the institutional Committee for Ethics in Animal Experimentation. Macrophages were harvested from the peritoneal cavities of mice and were resuspended in Hanks' balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, MO). The number of viable cells was determined by trypan blue dye exclusion. The resuspended macrophages (2×10^5 cells/well) were allowed to adhere to round 13 mm glass coverslips placed in 24-well plates (Corning

Inst., NY, US). Non adherent cells were removed by rising the coverslips with HBSS and the adherent cells were incubated in 1 ml of Roswell Park Memorial Institute (RPMI-1640) medium (Sigma) supplements with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere. After 48 h the cells were washed three times with serum-free RPMI 1640 medium and incubated for 1 or 3 h in the presence of 10^{-14}-10^{-7} M AII (Sigma) alone or with different concentrations 10^{14}-10^{-4} M) of losartan (DuPont de Nemours & Co., Wilmington, DE). The AII and losartan solutions were prepared in serum-free RPMI 1640 medium. Control cultures were incubated with serum-free RPMI-1640 medium.

Opsonized zymosan - Twenty milligrams of zymosan A (Sigma) dissolved in 1 ml of PBS (phosphate-buffered saline, pH 7.4) were added to 3 ml of pooled normal mouse serum and incubated for 15 min at 37°C with mixing. The particles were washed three times with PBS and resuspended in the same solution for the phagocytic assays.

Phagocytic assay - Macrophage monolayers were obtained as described above and the coverslips were washed with HBSS. Opsonized zymosan particles were added to the monolayers in a 5:1 (particle: macrophage) ratio and the coverslips incubated at 37°C in a 5% CO_2 humidified atmosphere. After 30 min, the coverslips were washed with HBSS, fixed in methanol, and stained with Giemsa. After drying, the coverslips were mounted on glass slides and examined microscopically. The percentage of cells with ingested particles was multiplied by the average number of particles per macrophage to calculate phagocytic index. At least 100 macrophages were counted per coverslip.

Dimethylthiazoldiphenyltetrazolium bromide (MTT) assay - The tetrazolium reduction assay was done as described by Denizot and Lang (1986). Briefly, macrophages were incubated for 4 h with 1 ml of serum-free medium containing 2 mg of MTT/ml (Sigma). The culture medium was removed and the blue formazan product obtained was solubilized in 1 ml of ethanol with shaking for 15 min on a microtiter plate shaker. The resulting absorbance was measured at 570 nm.

Nucleic acid content - The number of cells in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. (1991). The cells were washed twice with cold PBS and a soluble nucleotide pool was extracted with cold ethanol. The cells monolayers were then dissolved in 0.5 M NaOH for 1 h at 37°C, and the absorbance of the lysate was used as an index of the number of cells. The results were expressed as a percentage of the absorbance at 260 nm obtained for the controls.

Superoxide anion production - Superoxide production by the macrophages was measured by the reduction of ferricytochrome C (Sigma) as described by Pick and Mizel (1981). Peritoneal cells (2 x 105 cells/well) were incubated with reaction solution containing 80 µM ferricytochrome C with zymosan particles in a 5:1 (particle: macrophage) ratio in HBSS. The amount of ferricytochrome C reduced by superoxide was measured spectrophotometrically at 550 nm. Wells containing medium and ferricytochrome C only were used as controls. The total superoxide anion production was calculated using the formula:

Superoxide (nmoles) = A_{550} x [volume of incubation mixture (ml)] x 47.4 (Absolom 1986), where A_{550}, is the peak height or maximum absorbance at 550 nm. Superoxide generation was calculated by assuming that a change in absorbance of 1.0 at 550 nm corresponded to the presence of 47.4 nmol of superoxide. The extinction coefficient for ferricytochrome C at 550 nm was considered to be 21,000 $M^{-1} . cm^{-1}$.

Preliminary experiments showed that superoxide production by peritoneal cells was completely inhibited by the presence of superoxide desmutase. After the removal of non-adherent cells, the macrophages were covered with 1 ml per well of 1 N NaOH and incubated overnight at 37°C in a humidified incubator. The following day, the NaOH digest from all wells was pooled and the protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. The mean protein content (mg) per well determined in this assay was used to express the superoxide production by the cells, and was expressed as nmoles of superoxide produced per mg of protein per hour.

Immunofluorescence staining - Coverslips with macrophage monolayers were fixed in methanol (20°C) for 1 h. The cells were washed with cold PBS and non-specific binding sites were blocked with milk (3%) for 1 h at room temperature. After extensive washing, the cells were incubated overnight (4°C) with anti-AT_1 rabbit IgG (Santa Cruz) or anti-AT_2 goat IgG (Santa Cruz) at a dilution of 1:50. The cells were then washed with PBS and incubated for 2 h at room temperature with anti-rabbit IgG FITC (1:100) and anti-goat IgG FITC (1:200). The coverslips were mounted with antifading Vectashield media. The immunofluorescence staining was examined in a Bio-Rad scaning laser confocal microscope equipped with an Axovert 100 microscope (Boer-Lima et al. 2002).

Statistical analysis - Where appropriate, the results were expressed as the mean ± SEM. Statistical significance was assessed by ANOVA for repeated measurements followed by the Tukey post-hoc test to determine the extent of the differences using the SAS (Statistical Analysis System, version 6.12). A significance level of 5% was chosen.

RESULTS

Phagocytic assay - The effect of AII on the phagocytic activity of macrophages was evaluated over the concentration of 10^{-14} to 10^{-4}M. Exposure to 10^{-13} M and 10^{-12} M AII significantly potentiated ($p < 0.05$) zymosan-induced phagocytosis after 1 and at 10^{-12} M AII after 3 h in a concentration-dependent fashion (Fig. 1). Fig. 2 shows that losartan, a AII receptor antagonist, blocked the stimulatory effect of 10^{-12} M AII on phagocytosis up to 10^{-14} M AII concentration tested, with complete inhibition occurring at 10^{-6} M losartan ($p < 0.001$) (Fig. 1C). When the incubation time was increased to 3 h, losartan alone inhibited phagocytosis at concentrations ranging from 10^{-12} M to 10^{-4} M. Similar results were observed in the presence of 10^{-12} M AII, with a major effect at 10^{-6} M of losartan ($p < 0.001$) (Fig. 1D).

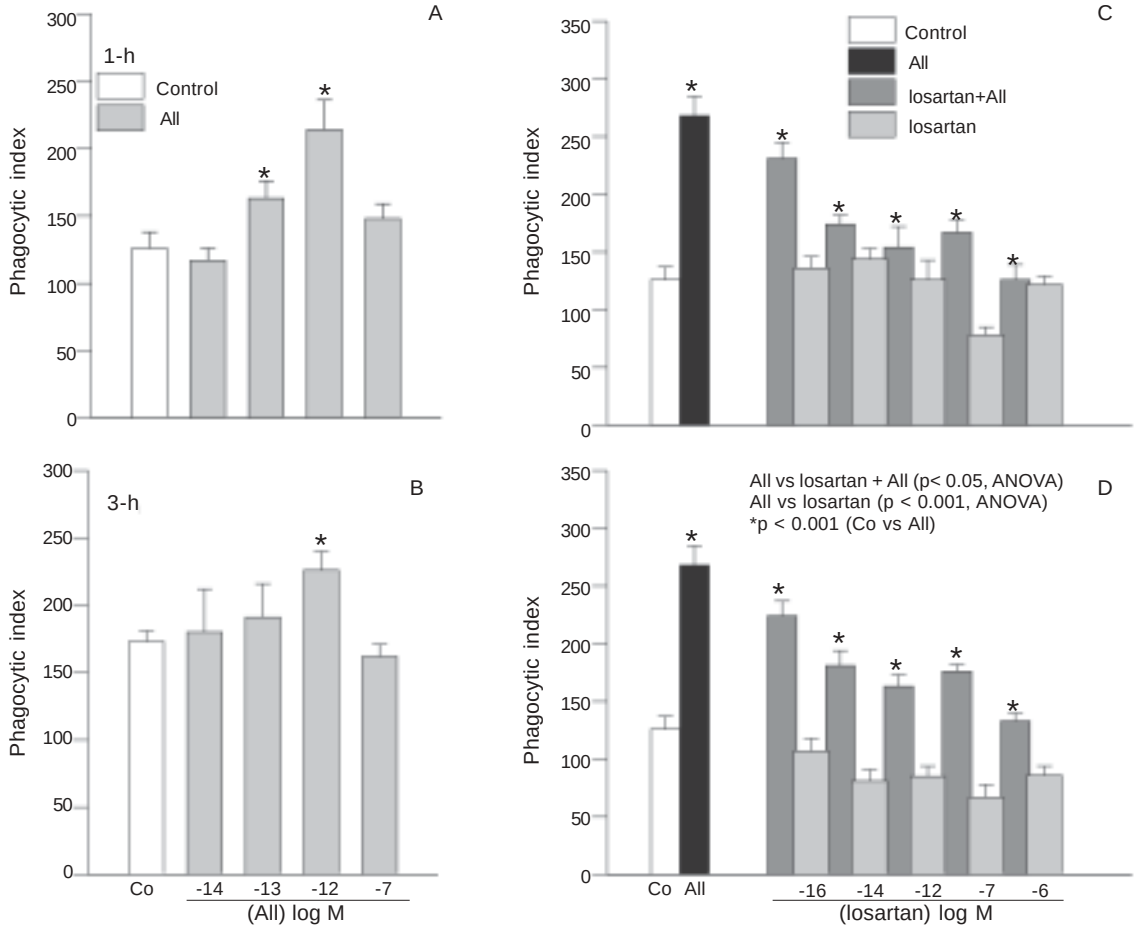

Fig. 1: dose-dependent effect of angiotensin II and/or losartan on the phagocyte activity of peritoneal macrophages. Peritoneal macrophages were exposed in vitro to various concentrations of AII (n = 9), losartan and AII (10^{-12} M, n = 9) + losartan for 1 h (A and C, n = 9 for each experimental group) or 3 h (D, n = 9), and then incubated with zymosan solution for 30 min to determine phagocytic activity. Control cells (Co) were incubated with Roswell Park Memorial Institute (RPMI-1640) medium alone. Statistical significance was assessed by ANOVA for repeated measurements followed by the Tukey post-hoc test to determine the extent of the differences. The data are expressed as means ± SEM. Two hundred cells were counted per sample.

Cytotoxicity assays - To assess whether the inhibitory effect of losartan on macrophage phagocytic activity was due to the blockade of AT_1 receptors, citotoxicity assays were done using the same losartan concentrations as described in the experiments above. No toxic effect was observed when macrophages were incubated with losartan at any of the concentrations tested (Fig. 2). Surprisingly, however, an increase in the nucleic acid content was observed after a 1 h incubation with 10^{-4} M losartan (Fig. 2A).

Superoxide anion production - The concentration of AII (10^{-12} M) that stimulated phagocytosis and this of losartan (10^{-6} M), which inhibited phagocytosis, did not significantly influence the superoxide production by macrophages, even after a 3 h exposure to the drugs (Fig. 2C).

Immunofluorescense - Immunohistochemistry gave a positive reaction for AT_1 and AT_2 receptors. Pretreating the cells with 10^{-12} M AII increased the intensity of AT_1 receptor staining (Fig. 3B). In contrast treatment with 10^{-6} M losartan or with AII + losartan decreased the number of receptors stained (Fig. 3C, D). Similar results were observed with AT_2 receptors when the cells were treated with 10^{-12} M AII (Fig. 3F).

DISCUSSION

Components of the RAS are present in isolated liver granulomas associated with murine schistosomiasis, and granuloma macrophages have receptors for AII. These observations, along with other evidence (Iwai et al. 1996), suggest that angiotensin II has a role in immunoregulation. Since AII can stimulate actin-myosin interactions and since macrophages have contractile proteins, the ability of AII to alter the phagocytic activity of granuloma macrophages was examined (Weinstock & Blum 1987, Iwai et al. 1996, Nickening et al. 1997, Lamperter et al. 1998). At concentrations, which saturate the AII receptors, AII enhanced the phagocytic activity of opsonized and unopsonized sheep red blood cells (RBC). Using a DNase inhibitory assay, similar concentrations of AII were found to intracellular actin polymerization. Cytochalasin B, which

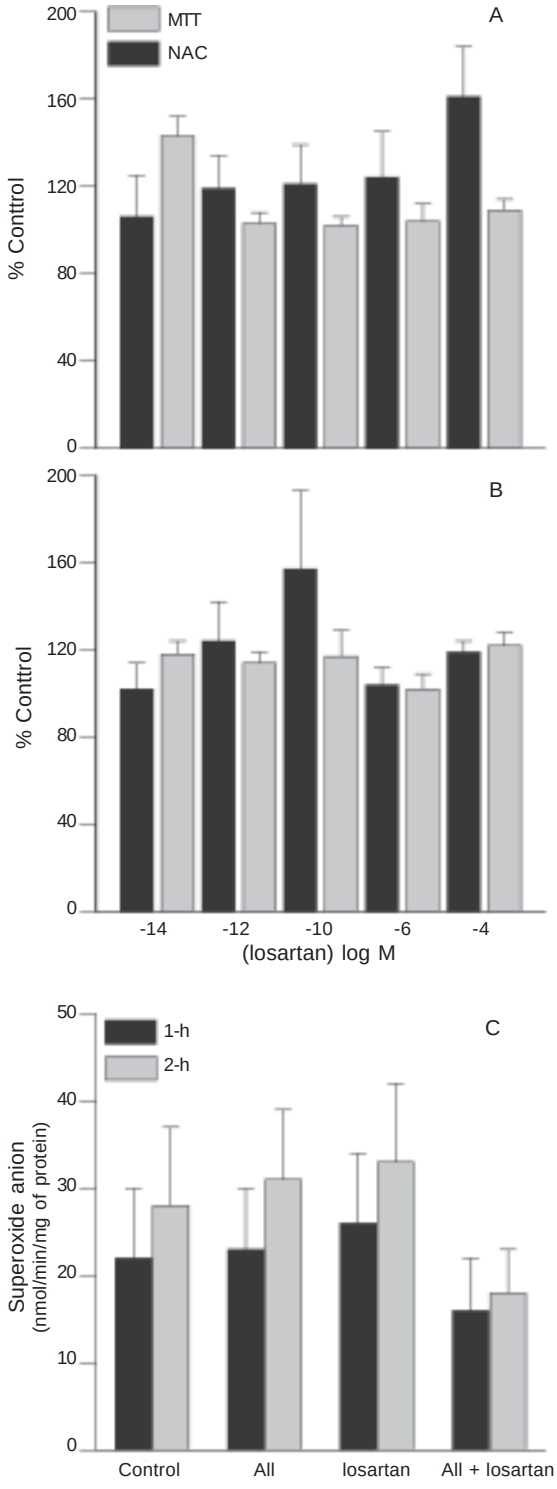

Fig. 2: macrophage viability evaluated by the nucleic acid content (NAC) and MTT (dimethylthiazoldiphenyltetrazolium bromide) reduction assay, and superoxide anion release by peritoneal macrophages (C) after treatment with AII (10^{-12} M, n = 8), losartan (10^{-6} M, n = 8) and angiotensin II + losartan (n = 8 for each time) for 1 h (A) or 3 h (B). The data are reported as the mean ± SEM of two independent experiments each run in quadruplicate. Statistical significance was assessed by ANOVA test for repeated measurements. A P value ≤ 0.05 was considered to indicate statistical significance. The data were expressed as a percentage of the corresponding control value.

impedes actin filament interactions, inhibited AII-stimulated phagocytosis AII analogs with little agonist activity blocked AII-stimulated actin polymerization and the phagocytosis of non-opsonized RBC and of RBC opsonized with IgG. These data support the contention that AII binds to AII receptors on macrophages and stimulates phagocytosis possibly through the polymerization of intracellular actin. AII of the components of the RAS, except for chymase, have been demonstrated in macrophages (Okamura et al. 1999). AII is involved in rosette formation and in the phagocytosis associated with the contractile elements of the cell, thereby affecting the cytoskeleton (Dezso & Fóris 1981, Fóris et al. 1983, Weinstock & Blum 1987, Dezso et al. 1988). Our results are consistent with these data and suggest that the RAS may have an important function in the phagocytic activity of macrophages. The concentration response curve for angiotensin II induced phagocytosis was bimodal, with the greatest stimulation at 10^{-13} M and 10^{-12} M AII concentrations tested.

To determine whether this stimulation occurred via AT_1 receptors, the cells were incubated with the AT_1 antagonist losartan prior to the determination of phagocytosis. A decrease in phagocytic activity was observed after a 1 h incubation with 10^{-6} M losartan. When the cells were incubated with different concentrations of losartan in the presence of 10^{-12} M AII there was a decrease in the phagocytic index, thus demonstrating the blockade of AT_1 receptors and their involvement in phagocytosis. When the incubation was increased to 3 h, losartan alone inhibited phagocytosis in the concentration range of 10^{-12} to 10^{-6} M, while in the presence of 10^{-12} M AII, concentrations up to 10^{-14} M of losartan tested also effectively prevented AII-induced increases in phagocytic function. This inhibitory action of losartan was not due to cytotoxicity since losartan did not affect cell viability as assessed by the MTT reduction and nucleic acid content. These results indicate that the binding of AII to AT_1 receptors is essential for the activation of phagocytosis in macrophages.

In addition of phagocytic activity, the effect of AII and losartan on the production of superoxide anions was investigated using ferricytochrome C reduction. Mesangial cells exposed to AII (10^{-7} M to 10^{-5} M) show a significant increase in superoxide production mediated by AT_1 receptors (Jaimes et al. 1998). In peritoneal macrophages, the concentrations of AII and losartan which stimulated and inhibited phagocytosis, respectively, did not influence superoxide production, perhaps because the concentrations tested were not optimal.

Immunofluorescense microscopy identified AT_1 and AT_2 receptors on peritoneal macrophages treating the cells with AII and losartan changed the immunoreactivity with AII increasing the number of AT_1 and AT_2 receptors, whereas losartan decreased the intensity of the immunofluorescense. Together, these results suggest that AII receptors may modulate murine macrophage activity and phagocytosis is dissociated from free-radical generation.

Perspectives - Our results support the hypothesis that angiotensin can directly modulate peritoneal macrophage function, and may have a therapeutic role as an immunomodulatory agent in modifying the host resistance to infection.

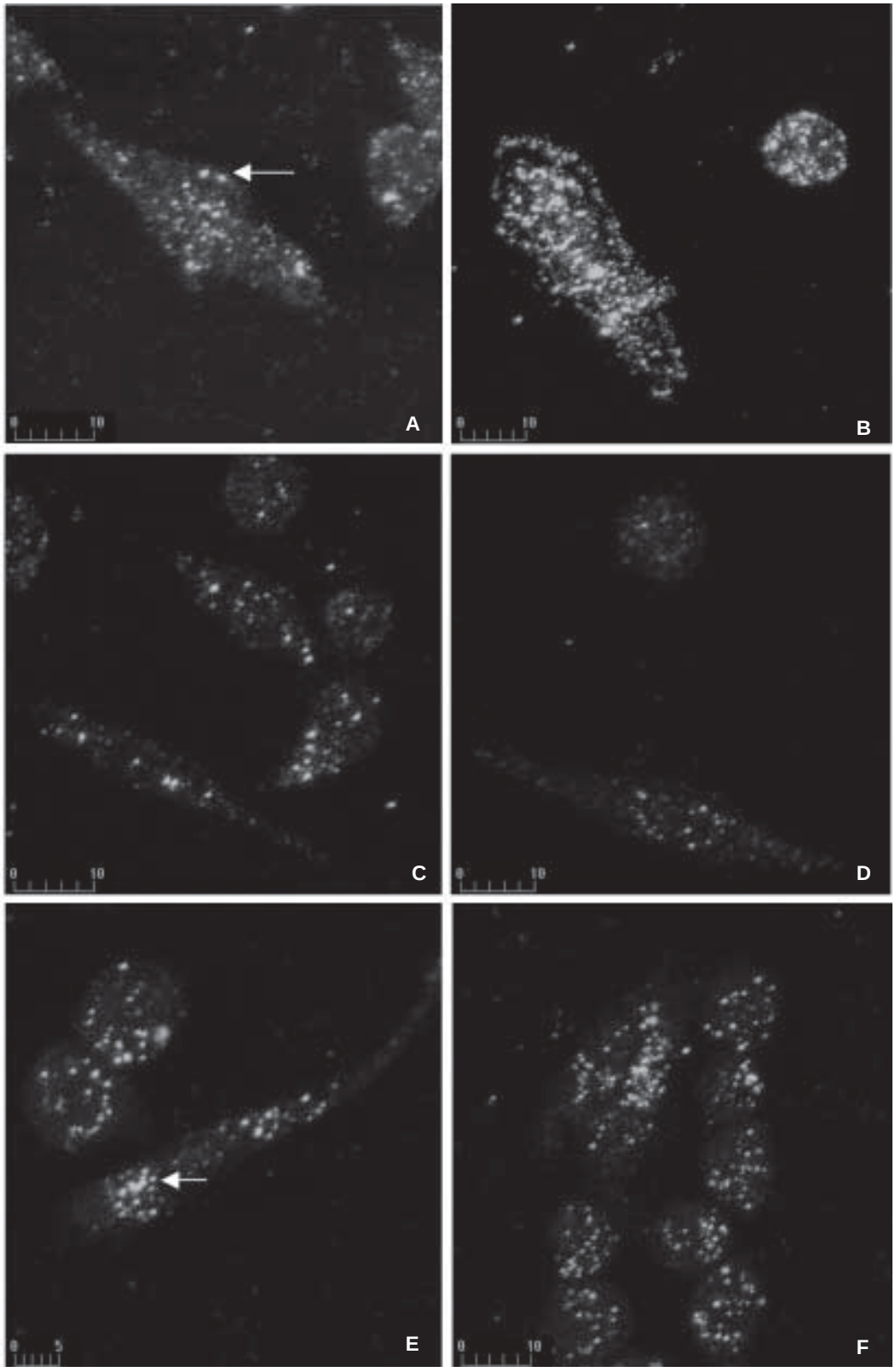

Fig. 3: immunoreactive AT_1 receptors in mouse peritoneal macrophages. A: control group; B: macrophages treated for 1 h with angiotensin II (10^{-12}M); C: macrophages treated for 1 h with losartan (10^{-6}M); D: macrophages treated for 1 h with AII and losartan. Immunoreactive AT_2 receptors in mouse peritoneal macrophages (E, F); E: control group; F: macrophages treated for 1 h with AII (10^{-12} M).

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