Different responses of the MIO-M1 Mueller cell line to angiotensin II under hyperglycemic or hypoxic conditions

ANSGAR BEUSE[1*], HEIDRUN L. DEISSLER[2*], MARGRIT HOLLBORN[1], JAN DARIUS UNTERLAUFT[1,3], CATHARINA BUSCH[1*] and MATUS REHAK[1,2*]

[1]Department of Ophthalmology, University of Leipzig, D-04103 Leipzig; [2]Department of Ophthalmology, Justus-Liebig-University Giessen, D-35392 Giessen, Germany; [3]Department of Ophthalmology, University of Bern, 3010 Bern, Switzerland

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Abstract. Members of the renin-angiotensin aldosterone system (RAAS) are expressed by various retinal tissues including Mueller glial cells. As the RAAS is hypothesized to play an important role in the pathogenesis of diseases that threaten vision, such as diabetic macular edema or retinal vein occlusion, the possible changes induced by exposure of the human cell line MIO-M1, an established model of Mueller cells, to angiotensin II or aldosterone for 6 h under hypoxic and/or hyperglycemic conditions were investigated. The mRNA expression levels of the members of the RAAS were assessed by reverse transcription-quantitative PCR, and the secretion of cytokines was assessed by ELISA. Under hyperglycemic conditions, the mRNA expression levels of the angiotensin-converting enzyme 2 (ACE2), angiotensin II receptors, AT_1 and AT_2, and the receptor of angiotensin (1-7) MAS1 were significantly higher after exposure to angiotensin II, and the expression of ACE2, AT_2, and IL-6 (a marker of inflammation) was significantly increased after treatment with aldosterone; the expression of the other targets investigated remained unchanged. Significantly more IL-6 was secreted by MIO-M1 cells exposed to hyperglycemia and angiotensin. When cells were cultured in a hypoxic environment, additional treatment with aldosterone significantly increased the mRNA expression levels of ACE, but significantly more ACE2 mRNA was expressed in the presence of angiotensin II. Under hypoxic plus hyperglycemic conditions, significantly less ACE but more AT_2 was expressed after treatment with angiotensin II, which also led to strongly elevated expression of IL-6. The mRNA expression levels of the angiogenic growth factor VEGF-A and secretion of the encoded protein were notably increased under hypoxic and hypoxic plus hyperglycemic conditions, irrespective of additional treatment with angiotensin II or aldosterone. These findings suggest that angiotensin II induces a pro-inflammatory response in MIO-M1 cells under hyperglycemic conditions despite activation of the counteracting ACE2/MAS1 signaling cascade. However, hypoxia results in an increased expression of angiogenic VEGF-A by these cells, which is not altered by angiotensin II or aldosterone.

Correspondence to: Dr Heidrun L. Deissler, Department of Ophthalmology, Justus Liebig University Giessen, Friedrichstrasse 18, D-35392 Giessen, Germany
E-mail: heidrun.deissler@augen.med.uni-giessen.de

*Contributed equally

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; AT_1, angiotensin II receptor 1; AT_2, angiotensin II receptor 2; DME, diabetic macula edema; DR, diabetic retinopathy; IL-6, interleukin-6; RAAS, renin-angiotensin aldosterone system; RVO, retinal vein occlusion; VEGF-A, vascular endothelial growth factor-A; VEGFR, VEGF receptor

Key words: diabetic macular edema, Mueller cells, renin-angiotensin aldosterone-system, angiotensin II, aldosterone

Introduction

Diabetic macula edema (DME) is a serious complication of diabetic retinopathy (DR) occurring in ~3.7% of DR patients, and, based on the estimated worldwide prevalence of diabetes of 5.4% by 2025 an exponential growth of DME and associated loss of vision is expected (1,2). In addition, retinal vein occlusion (RVO) results in hypoxia of the retina and eventually in the development of macular edema (3). Elevated permeability of the retinal endothelium and a decreased re-uptake of fluid by Mueller cells, which express multiple ion- and water-channels (such as Na^+-, K^+-, and aquaporin channels) leads to the accumulation of fluid in the macula (4). Decreased expression of ion channels results in deregulated trans-glial water transport and as a consequence in the swelling of retinal glial cells (4). Vascular endothelial growth factor A (VEGF-A), higher levels of which are present in the vitreous of patients with DR, DME, or RVO, elevates the permeability of the retinal endothelium, playing a key role in the pathophysiology of macular edema (3-7). Higher concentrations in the vitreous or aqueous humor of inflammatory cytokines, such as interleukin (IL)-6 and IL-1β are not only associated with the pathogenesis of RVO, respectively, but also with the development of DME and proliferative DR (8-12). Additionally, the ocular renin-angiotensin aldosterone-system (RAAS) also regulates the retinal

blood and fluid balance; several studies point to its major role in the development of DME (13-15). Members of the RAAS, including angiotensinogen (AGT), angiotensin-converting enzyme (ACE), and ACE2 as well as the receptors of angiotensin II, AT_1, and AT_2, encoded by the genes AGTR1 and AGTR2, respectively, and the G-protein-coupled receptor MAS1 of angiotensin (1-7), are expressed by retinal tissues including Mueller cells and retinal vessels (16-25). AGT is cleaved by the protease renin to give rise to angiotensin I and cleavage of the decapeptide by ACE in turn results in the vasoconstrictive octapeptide angiotensin II. This peptide hormone not only induces pro-inflammatory responses but may also be involved in angiogenesis, and these processes are mediated by its receptor AT_1 (13,25). Through its weakly expressed alternative receptor AT_2, the actions of angiotensin II can be counteracted (25,26). The G-protein-coupled receptor MAS1 induces anti-angiogenic and anti-inflammatory processes not only systemically but also in the retina; its ligand, the vasodilator angiotensin (1-7), is formed by the proteolytic removal of the C-terminal phenylalanine of angiotensin II by ACE2 (24,27).

Taken together, upregulation of AGT can lead to increased production of angiotensin II resulting in higher local RAAS activity in general; however, the consequences of its actions on Mueller cells and the retina in its entirety are not fully understood. Thus, the influence of angiotensin II or aldosterone on the expression of different mediators of DME or RVO pathogenesis, as well as the components of the RAAS under hypoxic and hyperglycemic conditions in MIO-M1 cells, a model of human Mueller cells, were investigated (28,29).

Materials and methods

Culture and treatment of MIO-M1. The spontaneously immortalized human Mueller glial cell line MIO-M1 (RRID: CVCL_0433) was purchased from University College London (28). Cells were cultured in DMEM (cat. no. 21885025, Thermo Fisher Scientific, Inc.) containing 5 mM glucose, and supplemented with 10% FBS, glutamax II, and penicillin/streptomycin (all purchased from Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. To confirm absence of mycoplasma, fixed MIO-M1 cells were regularly stained with DAPI ($\lambda_{excitation}/\lambda_{emmission}$=359 nm/461 nm) and evaluated using fluorescence microscopy, which would have enabled the detection of non-nuclear DNA, which would have indicated the possible presence of mycoplasma. To study the changes induced by 30 mM glucose (Carl Roth), 10 nM angiotensin II (cat. no. 05-23-0125, MilliporeSigma), 10 nM aldosterone (cat. no. A9477, MilliporeSigma), and hypoxia (0.1% O_2) or their combinations, $4x10^4$ MIO-M1 cells were seeded per well of a 12-well cell culture plate (Greiner Bio-One) in 1 ml DMEM supplemented with 10% FBS, glutamax II, and penicillin/streptomycin. When ~90% of the cell culture surface was covered by a monolayer of cells, the cell culture medium was replaced with serum-free DMEM. After further culture for 16 h, glucose, angiotensin II or aldosterone were added in a volume of 10 μl DMEM, and cells were incubated for an additional 6 h before cell culture supernatants and cells were harvested.

RNA isolation and cDNA synthesis. The InviTrap Spin Universal RNA Mini Kit (cat. no. 1060100200 Stratec Molecular) was used to isolate total RNA. The quality of the RNA samples was analyzed using a NanoDrop 1000 spectrophotometer (Peqlab). The A_{260}/A_{280} ratio was between 1.95 and 2.05 demonstrating a sufficiently good quality of the RNA samples. Possible contamination with DNA was removed with recombinant RNAse-free DNase I (cat. no. 4716728001, MilliporeSigma). cDNA synthesis was performed using 0.6 μg total RNA and a RevertAid H Minus First Strand cDNA Synthesis Kit, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.).

Quantitative (q)PCR. Semi-quantitative PCR was performed using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Inc.). The sequences of the primers used in the present study are listed in Table I. The amplification mixture (10 μl in total) contained 5 μl iQ™ SYBR® Green Supermix (cat. no. 170888x, Bio-Rad Laboratories, Inc.), specific primers (0.2 μM each), and 1 μl (~0.1 μg) of cDNA. The PCR amplification conditions were: Initial denaturation and enzyme activation at 95˚C, 3 min; followed by 45 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 20 sec, and extension at 72˚C for 45 sec. Each measurement included a melting curve analysis in the range of 65-95˚C with an increment of 0.5 K, and the length of the PCR products was determined by standard agarose gel electrophoresis. mRNA expression of actin (ACTB) was used for normalization and relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method: $\Delta Cq=Cq_{target\ gene}-Cq_{ACTB}$ and $\Delta\Delta Cq=\Delta Cq_{treatment}-\Delta Cq_{control}$ (30).

Determination of secreted VEGF-A or IL-6. The concentration of VEGF-A or IL-6 in cell culture supernatants of the treated MIO-M1 cells were determined using a Quantikine human VEGF-A ELISA kit (cat. no. DVE00, Bio-Techne) and a Quantikine Human IL-6 ELISA kit (cat. no. DVE6050, Bio-Techne), respectively. For measuring IL-6 levels, samples were diluted 1:10 in PBS without Ca^{2+}- and Mg^{2+}-ions (cat. no. 14190-169, Thermo Fisher Scientific, Inc.), and undiluted samples were used to determine the concentration of VEGF-A. Duplicate samples were processed according to the manufacturer's instructions and the analyte-dependent absorbance at 450 nm (reference wavelength: 570 nm) was measured 15 min after the addition of the stop solution with an Infinite 200Pro M Nano spectrophotometer controlled by Tecan I control software (version 2.0.10.0; Tecan Group, Ltd.). Standard curves (16 pg/ml to 1 ng/ml VEGF-A or 3-300 pg/ml IL-6) were always generated in parallel to the analysis of samples.

Statistical analysis. All experiments were performed at least three times. A one-way ANOVA followed by a Tukey's post-hoc test was used to compare the RT-qPCR signals from the treated cells and the ELISA results. For comparison of RT-qPCR signals from treated cells to the hypothetical value of 1 (normalized to the signal of control cells), a one-sample t-test was used, as in this type of statistical analysis, the variability of the values obtained from control cell signals is taken into consideration, although they appear without standard deviations (SD=0). $P<0.05$ was considered to indicate a

Table I. Sequences of the primers used for quantitative PCR.

Gene	Accession number	Forward primer, 5'-3'	Reverse primer, 5'-3'	Product size, bp
ACTB	NM_001101.5	ATGGCCACGGCTGCTTCCAGC	CATGGTGGTGCCGCCGAGCAG	237
IL6	NM_000600.4	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTTT	175
ACE	NM_001178057.1	TCAGCTACCTCGTCGATCAGT	TGTAAGGCACGCTAGAAGGAA	183
ACE2	NM_021804.3	GGGATCAGAGATCGGAAGAAGAAA	GGAGGTCTGAACATCATCAGTG	123
AGT	NM_000029.3	CTTTCAACACCTACGTCCACTTC	AGAAGTTGTCCTGGATGTCACTC	159
AGTR1	NM_032049.3	CAGTTTGCCAGCTATAATCCATC	TTCTTTAGGGCCTTCCAAATAAG	195
AGTR2	NM_000686.5	CTCTTCCTCTATGGGCAACCTAT	CAACACTCATGCAGGTGATAAAAA	134
MAS1	NM_002377.4	ACGTGACATCATTTGTTGTTGAG	AGTGAAGGGATTTCTTCTCATCC	188
VEGFA[a]	NM_001025370.3, NM_001287044.2, NM_003376.6	CCTGGTGGACATCTTCCAGGAGTA	CTCACCGCCTCGGCTTGTCACA	275, 407, 479
VEGFR2	NM_001110000.3	CTTCGAAGCATCAGCATAAGAAACT	TGGTCATCAGCCCACTGGAT	156

[a]Primer pairs were used to amplify the different splices variants of VEGF-A: VEGF-A_{121}, VEGF-A_{165}, and VEGF-A_{188}.

statistically significant difference. All statistical analyses were performed in GraphPad Prism version 9 (GraphPad Software, Inc.); means and standard deviations are provided as numbers or as scatter plots.

Results

Angiotensin II and aldosterone do not alter the expression of VEGF-A or VEGFR2 in MIO-M1 cells. Unchallenged MIO-M1 cells expressed or secreted considerable quantities of VEGF-A mRNA (Fig. 1A) or protein (Fig. 1B), respectively, and this was not significantly altered after treatment of the cells with 30 mM glucose, 10 nM angiotensin II, 10 nM aldosterone, or combinations thereof. Expression of VEGF-A mRNA and, accordingly, secretion of this growth factor was substantially and significantly higher after exposure of the cells to hypoxic or hypoxic plus hyperglycemic conditions. Again, these levels were not significantly altered by additional exposure of the cells to angiotensin II or aldosterone. Similarly, expression of VEGFR2 mRNA (Fig. 1C) remained stable. Only after culture of the cells under hypoxic plus hyperglycemic conditions, was its expression weakly but significantly higher.

Angiotensin II and aldosterone treatment alters the expression of RAAS members. When cells were cultured under standard conditions, the mRNA levels of members of the RAAS, AGT (Fig. 2A), ACE (Fig. 2B), the receptors of angiotensin II AT_1 (Fig. 2D) and AT_2 (Fig. 2E), and the receptor of angiotensin (1-7) MAS1 (Fig. 2F) were not significantly changed by additional treatment with angiotensin II or aldosterone. Exposure of the cells to 30 mM glucose did not alter their expression. However, the mRNA levels of AT_1 (Fig. 2D), AT_2 (Fig. 2E), and MAS1 (Fig. 2F) were elevated after treatment with angiotensin II under hyperglycemic conditions. These conditions also resulted in significantly enhanced expression of ACE2 (Fig. 2C), which also tended to be higher after angiotensin II exposure under normal conditions.

Cells were cultured under hypoxic conditions (at 0.1% O_2), which by itself did not affect the mRNA expression levels of the members of the RAAS (Fig. 3); however, additional treatment with angiotensin II significantly enhanced the expression of ACE2 mRNA (Fig. 3C) under these conditions. Angiotensin II-induced significantly higher mRNA expression of AT_2 when MIO-M1 cells were cultured under hypoxic plus hyperglycemic but not under hypoxic conditions (normalized expression levels were: 1.36±0.92 for hypoxia + angiotensin II compared to 6.14±3.47 for hypoxia plus hyperglycemia + angiotensin II, P=0.0029; n=6 per condition; Figs. 3E and 4E). The levels of AT_2 mRNA did not differ from the elevated levels observed when MIO-M1 cells were cultured in the presence of 30 mM glucose (normalized expression levels were: 3.94±1.47 for hyperglycemia + angiotensin II, n=5; compared to 6.14±3.47 for hypoxia plus hyperglycemia + angiotensin II, n=6 per condition; P>0.05; Figs. 2E and 4E). Under hypoxic plus hyperglycemic conditions, angiotensin II treatment also resulted in significantly reduced expression of ACE mRNA (Fig. 4B). Similar to angiotensin II, aldosterone-induced significantly higher levels of ACE2 mRNA (Fig. 2C) as well as of angiotensin II receptor AT_2 mRNA (Fig. 2E) levels under hyperglycemic conditions. Slightly, but significantly elevated levels of ACE mRNA (Fig. 3B) were observed after exposure of MIO-M1 cells to aldosterone under hypoxic conditions; the expression levels of the other targets remained unchanged throughout.

Angiotensin II alters the expression and secretion of IL-6 depending on the environment. As previously reported by others, unchallenged MIO-M1 cells secrete substantial amounts of the pro-inflammatory cytokine IL-6 (Fig. 5A) (31). However, neither its secretion (Fig. 5A) nor the expression of the corresponding mRNA (Fig. 5B) was altered

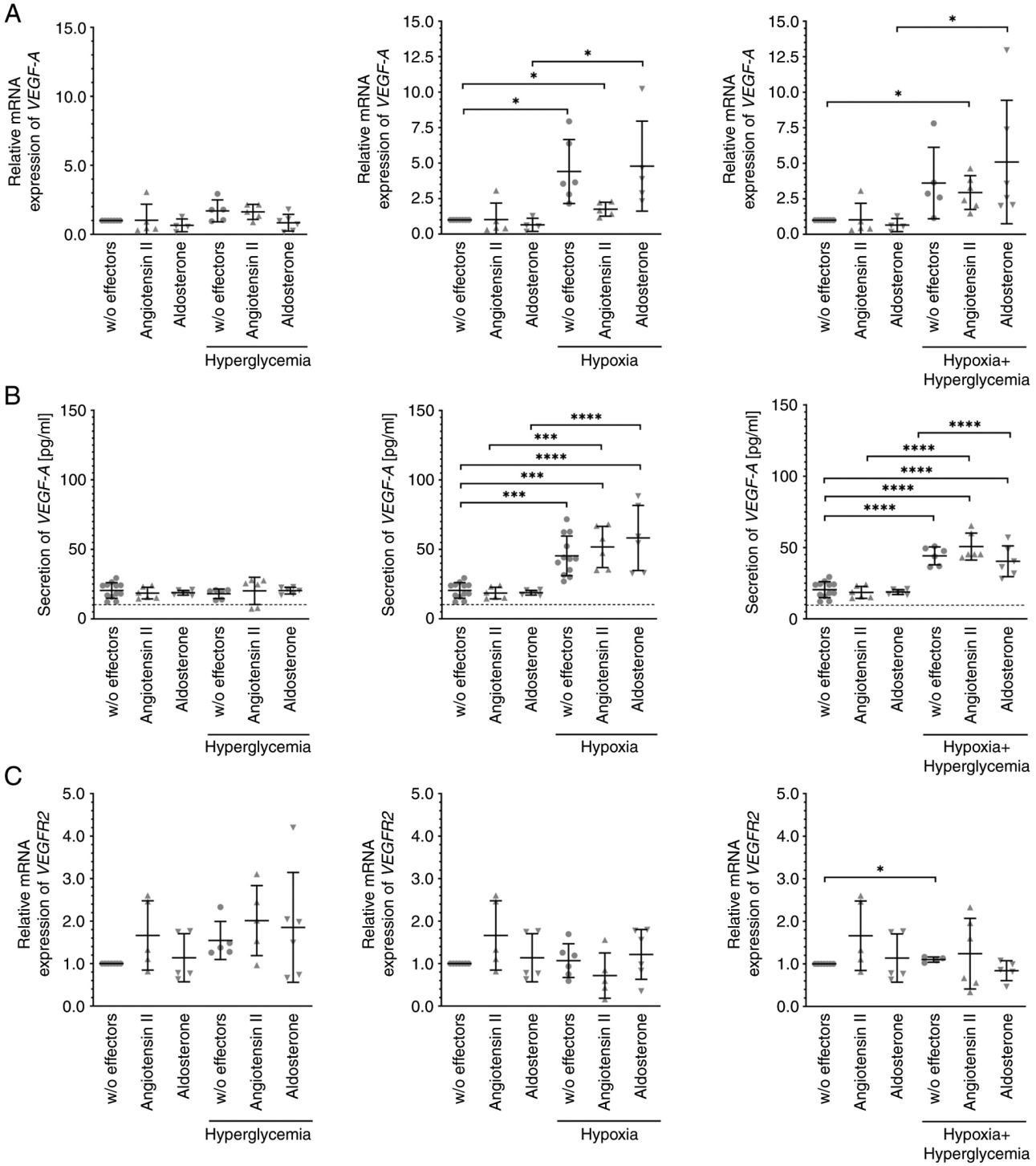

Figure 1. Exposure to hypoxia or hypoxia plus hyperglycemia increased the expression and secretion of VEGF-A and this was not modulated by angiotensin II or aldosterone. MIO-M1 cells were cultured under normal or hypoxic conditions in the absence or presence of 10 nM angiotensin II or 10 nM aldosterone for 6 h. Hyperglycemia was induced by addition of 25 mM glucose. mRNA expression levels of (A) VEGF-A and (C) VEGFR2 or (B) secretion of the growth factor were assessed. Hypoxia and hypoxia plus hyperglycemia, but not hyperglycemia alone, enhanced the (A) mRNA expression and (B) secretion of VEGF-A, which was not modulated by angiotensin II or aldosterone. (C) mRNA expression levels of VEGFR2 remained largely unchanged. Data were normalized to the relevant control. Data are presented as scatter plots depicting the mean ± standard deviation. *P<0.05, ***P<0.001, ****P<0.0001. Only statistically significant differences are indicated. The dotted line in (B) depicts the lowest detectable concentration. VEGF, vascular endothelial growth factor; VEGFR2, VEGFR receptor 2; w/o, without.

after exposure of the cells to hyperglycemia, hypoxia, or a combination of both. Both hormones also did not change IL-6 mRNA expression (Fig. 5D) in MIO-M1 cells cultured under normoxic or hypoxic conditions, and secretion (Fig. 5C) of the encoded protein also remained relatively unchanged. During exposure to angiotensin II under hyperglycemic conditions, MIO-M1 cells secreted significantly more IL-6 (Fig. 5C), and the mRNA expression (Fig. 5D) of the cytokine also tended to be higher. Under hypoxic plus hyperglycemic conditions, substantially and significantly more IL-6 mRNA

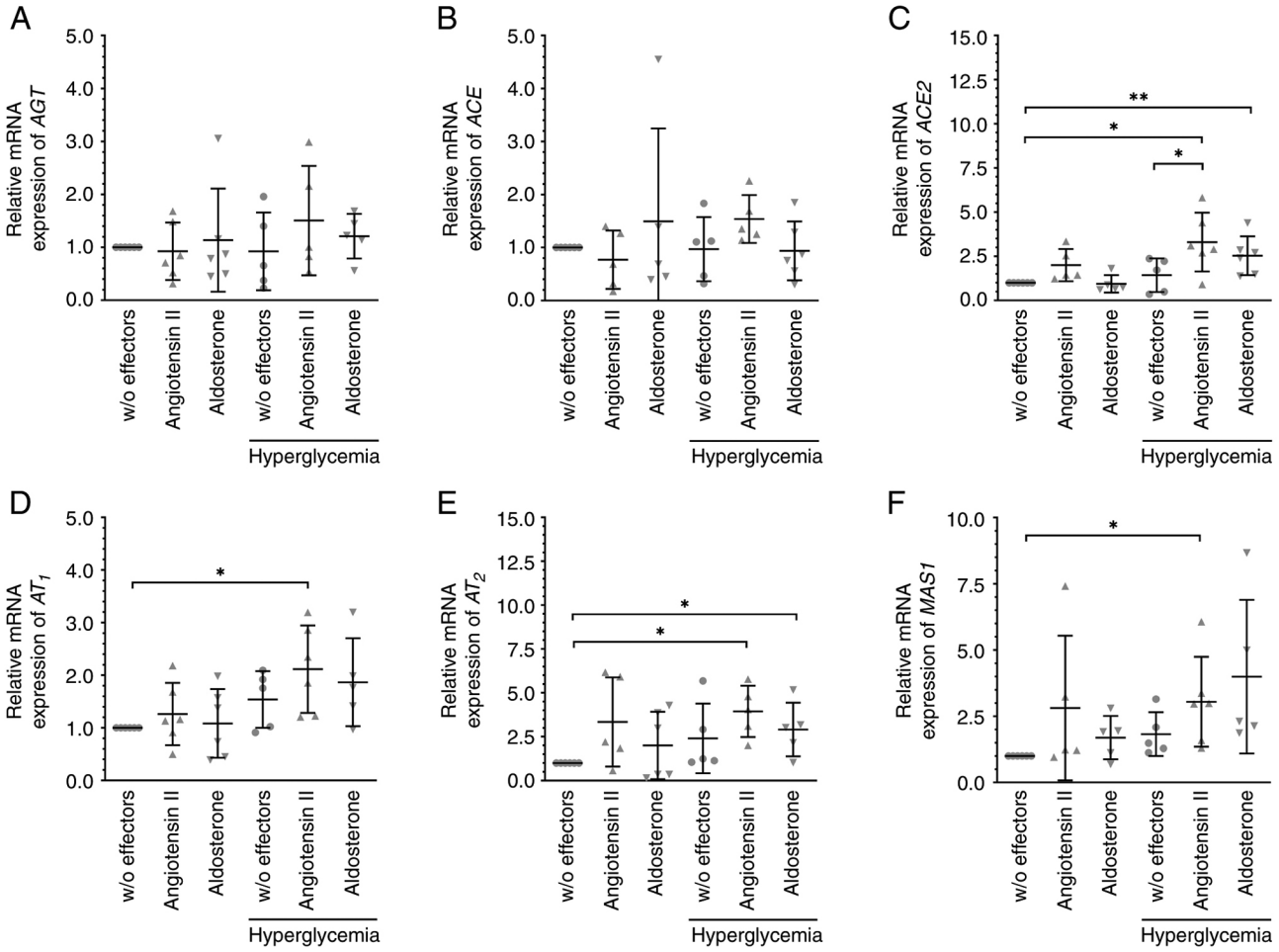

Figure 2. Angiotensin II and aldosterone altered the expression of members of the RAAS under hyperglycemic conditions. Cells were treated with 10 nM angiotensin II, 10 nM aldosterone or glucose (final concentration of 30 mM) to induce hyperglycemic conditions for 6 h. The mRNA expression levels of (A) AGT, (B) ACE, or (C) ACE2, (D) AT_1 and (E) AT_2, and (F) the G-protein-coupled receptor MAS1 were analyzed. Angiotensin II increased the mRNA expression levels of (C) ACE2, (D) AT_1, (E) AT_2, and (F) MAS1, and aldosterone those of (C) ACE2 and (E) AT_2. Data are presented as scatter plots depicting the mean ± standard deviation. *P<0.05, **P<0.01. RAAS, renin-angiotensin aldosterone system; AGT, angiotensinogen; ACE, angiotensin-converting enzyme; AT_1, angiotensin II receptor 1; AT_2, angiotensin II receptor 2; w/o, without.

(Fig. 5D) was expressed after additional exposure of the cells to angiotensin II and cells also secreted more IL-6 (Fig. 5C), although the difference was not significant. Aldosterone treatment of MIO-M1 cells under hyperglycemic conditions resulted in slightly, but significantly enhanced expression of IL-6 mRNA (Fig. 5D), but secretion of the cytokine (Fig. 5C) remained unchanged.

Discussion

Confirming previously published data, it was shown that the human cell line MIO-M1, an accepted model of human Mueller cells, expresses AGT, ACE, ACE2, angiotensin II receptors AT_1 and AT_2, as well as the receptor of angiotensin (1-7) MAS1 (17-19,21-24,28). The mRNA expression levels of these were largely unchanged when cells were exposed to hyperglycemic or hypoxic conditions or both. Exposure of cells to these conditions seemed to not result in induction of cellular stress, which may adversely affect the outcome of the investigations, since expression of IL-6 mRNA, a marker of cellular stress, remained stable. It could be suggested that an incubation time of 6 h is too short, but due to the short half-life of angiotensin II, longer exposure times would likely not result in more relevant data. However, expression and secretion of VEGF-A were substantially increased by hypoxia alone or in combination with hyperglycemia confirming the expected strong response of the cells to their altered environment within the studied time span. Angiotensin II and aldosterone did not modulate VEGF-A levels, proving the dominant role of hypoxia in the regulation of the growth factors' expression and secretion. It was to be expected that hyperglycemia alone did not modulate VEGF-A expression and secretion within 6 h, as possible changes likely manifest only after extended exposure (32). VEGF receptors are expressed in various retinal tissues including the retinal vasculature, Mueller cells, and the retinal pigment epithelium (33,34). Upregulation of VEGFR2 in the retinal vasculature is associated with the development of DR and its activation by the ligand VEGF-A_{165} results in elevated permeability of retinal endothelial cells or increased expression of pro-inflammatory mediators in Mueller cells *in vitro* (29,34,35). Inhibitors of ACE and/or AT_1, at least in part, prevent VEGF-A_{165}-induced permeability of retinal endothelial cells *in vitro* and *in vivo* as well as retinal

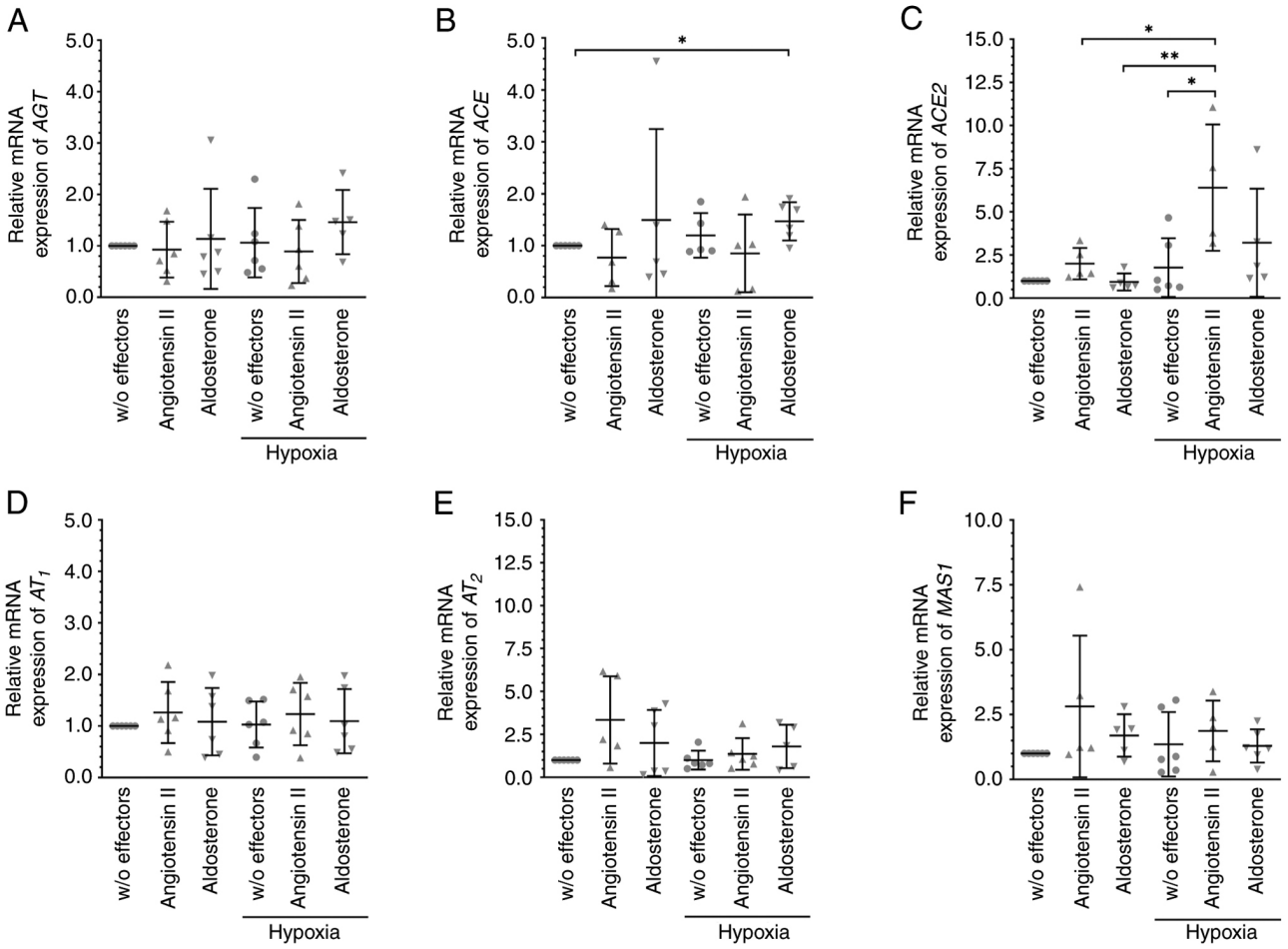

Figure 3. Angiotensin II and aldosterone treatment altered the expression levels of members of the RAAS under hypoxic conditions. To induce a hypoxic environment, MIO-M1 cells were cultured at 0.1% O_2 with 10 nM angiotensin II or 10 nM aldosterone for 6 h, and the mRNA expression levels of (A) AGT, (B) ACE, (C) ACE2, (D) AT_1, (E) AT_2 and (F) MAS1 were analyzed. Aldosterone slightly increased the expression of ACE, and angiotensin II increased the expression of ACE2. Data are presented as scatter plots depicting the mean ± standard deviation. *P<0.05, **P<0.01. RAAS, renin-angiotensin aldosterone system; AGT, angiotensinogen; ACE, angiotensin-converting enzyme; AT_1, angiotensin II receptor 1; AT_2, angiotensin II receptor 2; w/o, without.

neovascularization, thereby proving an interaction between both signaling pathways (36-39). However, expression and secretion of VEGF-A by MIO-M1 cells were not altered by angiotensin II likely reflecting the different behaviors of both cell types.

mRNAs coding for proteins of the RAAS indeed exhibited differential expression patterns in the presence of either angiotensin II or aldosterone when cells were cultured under hyperglycemic and/or hypoxic conditions. It is of interest, that angiotensin II did not alter the expression of its precursor AGT under any of the tested conditions, which may indicate that the peptide hormone cannot, directly or indirectly, induce its own expression in Mueller cells. To assess a possible pro-inflammatory response of Mueller cells, the changes in the expression of mRNA as well as secretion of the pro-inflammatory cytokine IL-6, which is constitutively expressed by this cell type (including MIO-M1 cells), was assessed (31). However, under normoxic or hypoxic conditions, the amounts of the secreted cytokine and its mRNA expression levels were not significantly altered by the treatment with angiotensin II or aldosterone, suggesting that the hormones do not induce a pro-inflammatory response of the cells under these circumstances. Interestingly, aldosterone significantly enhanced the expression of ACE mRNA under hypoxic conditions, thus not resulting in an inflammatory response, that is enhanced expression or secretion of IL-6 via the angiotensin II/AT_1-axis. Angiotensin II, on the other hand, increased the expression of ACE2, resulting in its own inactivation by the formation of angiotensin (1-7), which does not activate AT_1. Similar to the behavior of endothelial cells from the human umbilical cord, the mRNA expression levels of IL-6 were increased in MIO-M1 cells exposed to hyperglycemia and aldosterone, although the amount of the secreted cytokine remained unchanged from control cells (40). However, increased IL-6 expression is likely independent of the angiotensin II/AT_1-axis, as possibly endogenously produced peptide hormone is inactivated by high levels of ACE2.

Although angiotensin II did not significantly increase IL-6 mRNA expression in MIO-M1 cells cultured under hyperglycemic conditions, more IL-6 was secreted under these conditions. Higher expression of AT_1 mRNA could lead to stronger activation of the pro-inflammatory angiotensin II/AT_1 signaling cascade, similar to that observed for angiotensin II-activated retinal microglia, which express higher quantities of various pro-inflammatory cytokines and

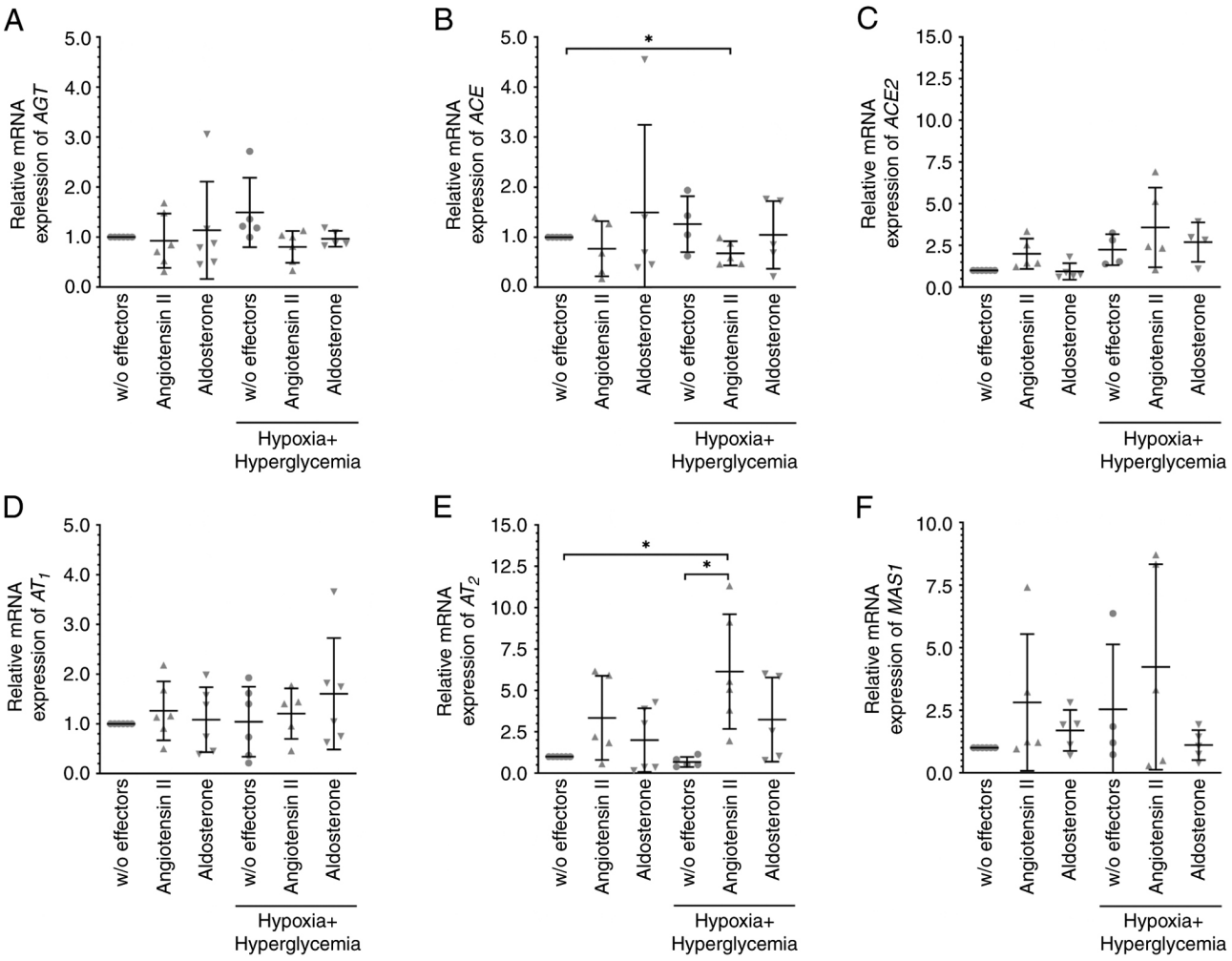

Figure 4. Angiotensin II, but not aldosterone altered the expression levels of members of the RAAS under hypoxic plus hyperglycemic conditions. MIO-M1 cells were cultured at 0.1% O_2 plus glucose (final concentration of 30 mM) with 10 nM angiotensin II or 10 nM aldosterone for 6 h, and the mRNA expression levels of (A) AGT, (B) ACE, (C) ACE2, (D) AT_1, (E) AT_2, and (F) MAS1 were analyzed. Angiotensin II lowered the levels of (B) ACE but increased those of (E) AT_2. Data are presented as scatter plots depicting the means ± standard deviation. *P<0.05. RAAS, renin-angiotensin aldosterone system; AGT, angiotensinogen; ACE, angiotensin-converting enzyme; AT_1, angiotensin II receptor 1; AT_2, angiotensin II receptor 2; w/o, without.

chemokines including IL-6, a process that is mediated by AT_1 (41). Elevated permeability of retinal endothelial cells due to IL-6-mediated trans-signaling *in vitro* likely contributes to the breakdown of the inner blood-retina barrier *in vivo* (42). The protective ACE2/angiotensin (1-7)/MAS1 signaling cascade is upregulated during acute and chronic diseases of the heart or kidney to counteract detrimental processes (43,44). This signaling cascade seems to also be activated by Mueller cells exposed to hyperglycemia and angiotensin II, as expression of ACE2 and MAS1 RNA was elevated. Thus, the concentrations of the vasodilator angiotensin (1-7) formed by protease ACE2 may be higher, and through its interaction with receptor MAS1, anti-angiogenic and anti-inflammatory processes can be induced (24-27). However, as MIO-M1 cells secreted increased quantities of IL-6 when exposed to hyperglycemia and angiotensin II, the pro-inflammatory axis seems to exceed the anti-inflammatory response. A similar inflammatory response to angiotensin II was also observed after additional exposure of the cells to hyperglycemia plus hypoxia, as the expression of IL-6 mRNA was substantially upregulated. However, the observed lower expression of ACE mRNA is in line with an assumed capacity of MIO-M1 cells to counteract angiotensin II-induced pro-inflammatory signaling.

In vivo, Mueller cells, retinal endothelial cells, and retinal pericytes form the so-called neurovascular unit, which tightly regulates vascular homeostasis in the retina (45). Whether the cellular interactions change their individual responses to angiotensin II could not be evaluated in the present study. However, inhibitors of ACE or AT_1 were found to, at least in part, improve the outcomes of DME in diabetic patients, which supports the findings of the present study that Mueller cells likely contribute to angiotensin II-mediated inflammatory responses present in the early development of this disease (46,47). In contrast, the impact of Mueller cells on angiotensin II-mediated inflammatory responses observed in the early development of RVO is likely low when hypoxia plays a dominant role accompanied by induction of expression and secretion of the angiogenic and permeability-inducing growth factor VEGF-A (9).

In conclusion, the results of the present *in vitro* study provide evidence that the responses of Mueller cells to activation of the RAAS by angiotensin II depend on the

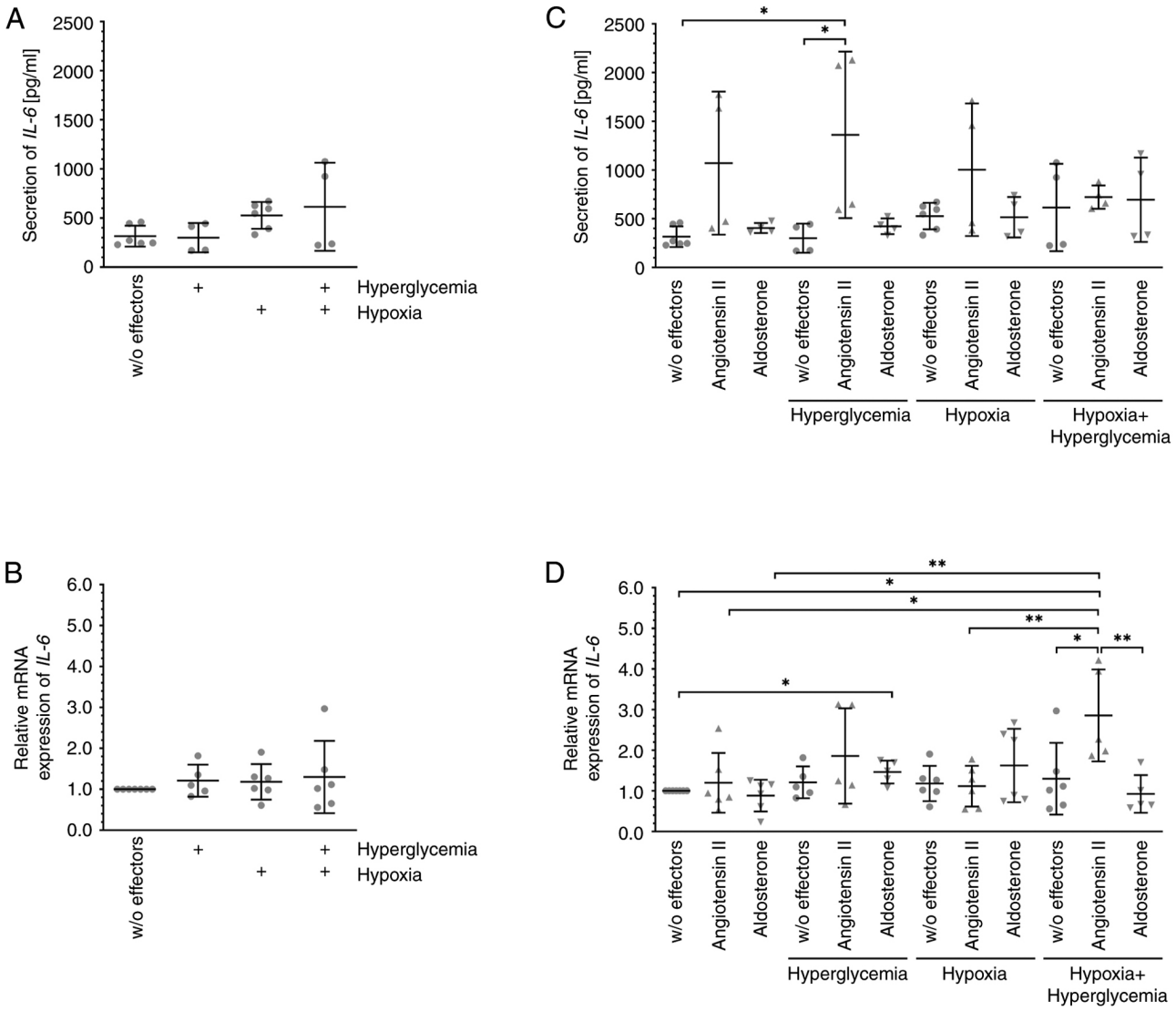

Figure 5. Exposure to hyperglycemia increased the secretion of IL-6 in the presence of angiotensin II. MIO-M1 cells were cultured under normoxic and/or hypoxic conditions in the absence or presence of 10 nM angiotensin II or 10 nM aldosterone for 6 h; hyperglycemia was induced by the addition of 25 mM glucose. (A and C) Secretion and (B and D) mRNA expression levels of IL-6 were assessed. (A) Secretion and (B) mRNA expression levels of IL-6 were not altered by exposure of the cells to hyperglycemia, hypoxia, or both. Angiotensin II significantly increased (C) the secretion of IL-6 when cells were cultured in a hyperglycemic environment and notably increased the (D) mRNA expression of the cytokine when cells were cultured under hypoxic plus hyperglycemic conditions. Data are presented as scatter plots depicting the means ± standard deviation. *P<0.05, **P<0.01. IL-6, interleukin-6; w/o, without.

environment: A pro-inflammatory response is observed under hyperglycemic (plus hypoxic) conditions, whereas changes induced by hypoxia are not modulated by angiotensin II.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CB and MR designed the general concept of the study; specific experimental conditions were established by AB, MH, HLD, and CB. AB and MH performed the experiments. AB, CB, MH, HLD, JDU curated and analyzed the data. AB, CB, MH, and HLD wrote the original draft manuscript. All authors reviewed and edited the manuscript. JDU and MH provided resources. All authors have read and approved the final manuscript. CB and HLD confirm the authenticity of the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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