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Factor XIIIA Transglutaminase Crosslinks AT_1 Receptor Dimers of Monocytes at the Onset of Atherosclerosis

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Summary

Many G protein-coupled receptors form dimers in cells. However, underlying mechanisms are barely understood. We report here that intracellular factor XIIIA transglutaminase crosslinks agonist-induced AT_1 receptor homodimers via glutamine[315] in the carboxyl-terminal tail of the AT_1 receptor. The crosslinked dimers displayed enhanced signaling and desensitization in vitro and in vivo. Inhibition of angiotensin II release or of factor XIIIA activity prevented formation of crosslinked AT_1 receptor dimers. In agreement with this finding, factor XIIIA-deficient individuals lacked crosslinked AT_1 dimers. Elevated levels of crosslinked AT_1 dimers were present on monocytes of patients with the common atherogenic risk factor hypertension and correlated with an enhanced angiotensin II-dependent monocyte adhesion to endothelial cells. Elevated levels of crosslinked AT_1 receptor dimers on monocytes could sustain the process of atherogenesis, because inhibition of angiotensin II generation or of intracellular factor XIIIA activity suppressed the appearance of crosslinked AT_1 receptors and symptoms of atherosclerosis in ApoE-deficient mice.

Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of cell surface membrane receptors and mediate a panoply of different physiological functions. Although GPCRs have long been considered to function as monomers, increasing evidence suggests that homo- and heterodimerization are characteristic features of this receptor family. Homo- and heterodimerization of GPCRs regulate many receptor-specific functions such as ligand binding, signaling, desensitization, and cell surface targeting (Franco et al., 2003; Terrillon and Bouvier, 2004). Yet, little is known about the pathophysiological relevance of GPCR dimerization in vivo.

We previously detected increased levels of AT_1/B_2 receptor heterodimers on platelets and vessels of preeclamptic women. These dimers seem to contribute to the enhanced angiotensin II responsiveness of this hypertensive disorder (AbdAlla et al., 2001b; Quitterer et al., 2004). An enhanced angiotensin II responsiveness is also a prominent feature of essential hypertension and of related cardiovascular disorders (Ljungman et al., 1983; Widgren et al., 1992). Moreover, the involvement of the angiotensin II-AT_1 system in the pathogenesis of essential hypertension and of vascular disease is well established (Schiffrin, 2002). Remarkably, hypertensive patients display an enhanced angiotensin II-dependent monocyte activation and adhesion to endothelial cells that are still not understood (Dörffel et al., 1999, 2001). Since an enhanced monocyte adhesiveness may sensitize hypertensive patients to the development of atherosclerosis (Dörffel et al., 2001; Dzau, 2001), we investigated mechanisms accounting for the enhanced AT_1 responsiveness of monocytes.

Receptor homo- and heterodimerization specifically modify signaling of individual receptor subtypes. Therefore, we sought to investigate the involvement of receptor dimerization in the enhanced angiotensin II responsiveness of monocytes. The AT_1 receptor forms heterodimers with various other receptors, such as the bradykinin B_2 receptor (AbdAlla et al., 2000), the angiotensin II AT_2 receptor (AbdAlla et al., 2001a), the β_2-adrenergic receptor (Barki-Harrington et al., 2003), and the dopamine D_1 receptor (Zeng et al., 2003). Heterodimerization of the AT_1 receptor with the B_2 receptor enhances specifically angiotensin II-stimulated signaling of preeclamptic patients (AbdAlla et al., 2001b). This event does not seem to contribute to the enhanced angiotensin II responsiveness of monocytes, because monocytes do not express significant amounts of B_2 receptors. The AT_1 receptor is also reported to form homodimers (AbdAlla et al., 2001a, 2001b). GPCR homodimerization may affect receptor signaling as well as receptor internalization or cell surface targeting. Analysis of the enhanced AT_1-dependent activation of monocytes led us to identify hyperresponsive crosslinked AT_1 receptor homodimers triggered by angiotensin II and the transglutaminase activity of intracellular factor XIIIA. We herein present strong evidence that the species of crosslinked AT_1 dimers contributes to an enhanced monocyte adhesiveness of hypertensive patients and thereby may sustain the process of atherogenesis by chronic sensitization of circulating monocytes.

Results

Monocyte Activation in Humans

To analyze mechanisms accounting for an enhanced AT_1-dependent monocyte activation in essential hypertension, we determined the release of interleukin-1β as an activation marker of circulating monocytes, enhancing monocyte-endothelial cell adhesion. The basal and the angiotensin II-stimulated release of interleukin-1β were significantly increased in hypertensive patients compared to the healthy controls (Figures 1A and 1B). The enhanced release of interleukin-1β was mediated by the angiotensin II AT_1 receptor, as evidenced by the inhibition with the AT_1-specific antagonist losartan (Figures 1A and 1B). For comparison, the release of interleukin-1β after stimulation of the monocytic ET_A receptor

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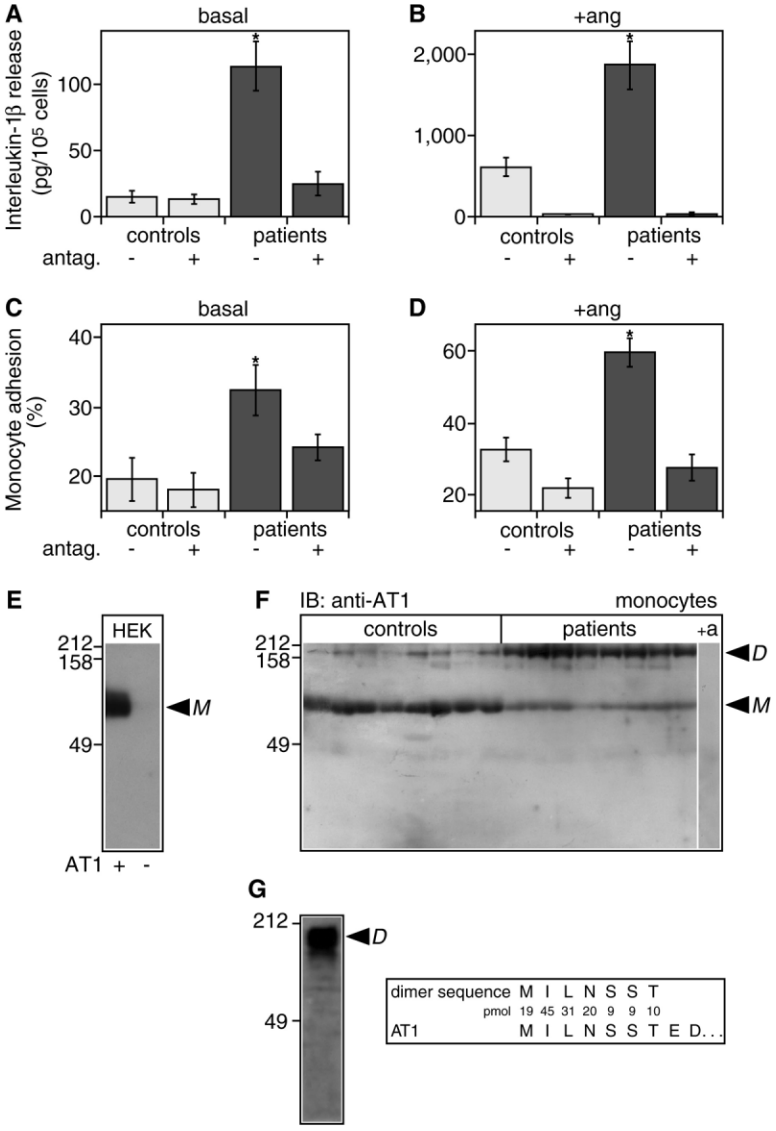

Figure 1. Increased Monocyte Activation and Covalently Bonded AT_1 Dimers of Hypertensive Patients

(A and B) Basal (A) and angiotensin II-stimulated (B, +ang, 10 nM) release of interleukin-1β from monocytes of eight healthy control individuals (controls) and eight hypertensive patients (patients). The AT_1-specific antagonist losartan (5 μM) was added as indicated (± antag.; *$p < 0.02$ versus controls).

(C and D) Basal and angiotensin II-stimulated adhesion of monocytes to endothelial cells determined with monocytes of the eight controls and of the eight patients (*$p < 0.02$ versus controls).

(E) Specificity of the antibodies for AT_1 was controlled with HEK cells expressing the AT_1 receptor (lane 1, +) or with mock-transfected cells (lane 2, −).

(F) Increased levels of SDS-stable AT_1 receptor homodimers on monocytes of the eight patients compared to the eight controls. The AT_1 receptor was detected in immunoblot of monocytic membranes with affinity-purified AT_1-specific antibodies (IB, anti-AT1). Arrowheads mark receptor monomers (*M*) and homodimers (*D*). The lane marked with +a is a specificity control performed in the presence of the corresponding antigen used for immunization.

(G) Immunoblot of affinity-purified monocytic dimeric AT_1 receptor isolated from patients (left panel). N-terminal protein sequencing of the protein yielded solely the AT_1 receptor sequence (right panel).

by endothelin-1 was not significantly different between controls and patients (312 ± 57 pg/10^5 cells, n = 8, versus 310 ± 63 pg/10^5 cells, n = 8; data not shown). Thus, there is an increased AT_1-stimulated interleukin-1β release from monocytes of hypertensive patients.

We further analyzed the AT_1-stimulated activation of monocytes and determined the adhesion of monocytes to endothelial cells. Basal and angiotensin II-stimulated monocyte adhesion of the patients was also significantly increased (Figures 1C and 1D). These findings are in agreement with previous data demonstrating an increased AT_1 receptor-dependent activation of monocytes in hypertension (Dörffel et al., 1999; 2001).

Identification of Covalently Dimerized AT_1 Receptors

The mechanism of the increased AT_1-dependent monocyte activation is not known. We assessed a relationship between enhanced AT_1 activity and AT_1 dimerization, because the enhanced AT_1 receptor responsiveness of another vascular disorder, preeclampsia, is due to altered AT_1/B_2 receptor heterodimerization (AbdAlla et al., 2001b). But AT_1/B_2 receptor heterodimerization does not seem to play a major role in enhancing monocyte activation, because monocytes do not express significant amounts of B_2 receptors (data not shown). We therefore focused on the AT_1 receptor protein and detected the monocytic AT_1 receptor with AT_1-specific antibodies in immunoblot (AbdAlla et al., 2000, 2001a, 2001b). The monocytic AT_1 receptor of the control individuals with an apparent molecular mass of M_r ~75–85 kDa was predominantly monomeric, as was the AT_1 receptor of transfected HEK cells (Figure 1F versus Figure 1E). In contrast, a major proportion of the monocytic AT_1 receptor of the patients had a high molecular mass of <200 kDa under the standard reducing conditions of SDS-PAGE supplemented with urea (Figure 1F). N-terminal protein sequencing of the affinity-purified high molecular mass AT_1 receptor isolated from patients exclusively yielded the N-terminal sequence of AT_1, *MILNSST* (Fig-

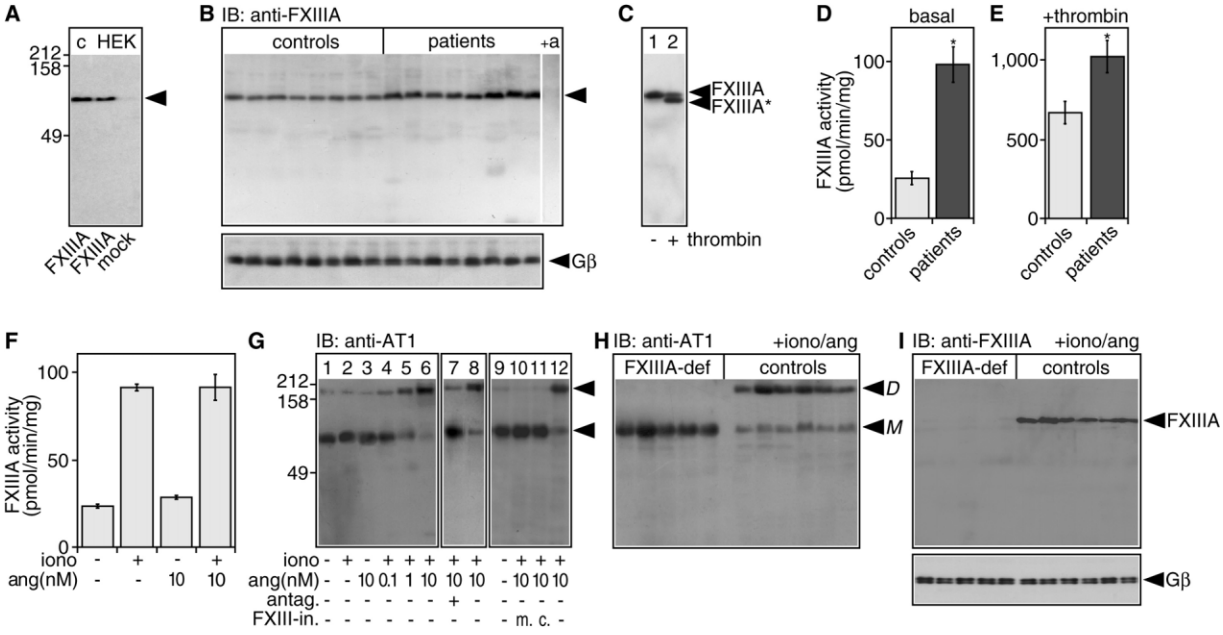

Figure 2. Intracellular Monocytic Factor XIIIA Transglutaminase Crosslinks AT_1 Dimers

(A) Immunoblot detection of purified factor XIIIA (c) or of factor XIIIA expressed in HEK cells (HEK) with affinity-purified factor XIIIA-specific antibodies. Mock-transfected cells served as a control.

(B) Monocytic factor XIIIA of the eight control individuals (control) and of the eight hypertensive patients (patients) used for the experiments in Figure 1. The amount of the loaded protein was controlled by anti-Gβ (lower panel). The lane marked with +a is a specificity control performed in the presence of the corresponding antigen used for immunization.

(C) Partial activation of factor XIIIA in the cytosolic fraction of monocytes by purified thrombin (−/+) followed by immunoblot detection.

(D and E) Basal (D) and thrombin-stimulated (E) monocytic factor XIIIA transglutaminase activity of the eight control individuals and of the eight patients assessed by a [^{3}H]putrescine incorporation assay (*$p < 0.01$).

(F) Ionomycin (iono, 0.1 μM, 8 hr) increased factor XIIIA transglutaminase activity of human monocytes in the absence and presence of angiotensin II (ang).

(G) Upon factor XIIIA activation by ionomycin, angiotensin II (ang, applied for 8 hr as indicated) induced the appearance of crosslinked AT_1 dimers in a concentration-dependent manner on moncytes of a control individual as determined in immunoblot with affinity-purified AT_1-specific antibodies (lanes 1–6). The AT_1-specific antagonist losartan (antag., 5 μM, lane 7 versus lane 8) or the transglutaminase inhibitors monodansyl cadaverine (m., lane 10) and cystamine (c., lane 11) at 200 μM suppressed the angiotensin II-induced formation of crosslinked AT_1 dimers (lane 12 versus lane 9).

(H) Monocytes of five patients with congenital factor XIIIA deficiency (FXIIIA-def) lack crosslinked AT_1 dimers (lanes 1–5), while AT_1 on monocytes of six age-matched controls is predominantly dimeric upon activation by ionomycin and angiotensin II (+iono/ang).

(I) Monocytes of factor XIIIA-deficient patients lack factor XIIIA, while factor XIIIA is detected in monocytes of the six control individuals. Equal protein loading was controlled by probing with anti-Gβ (lower panel).

ure 1G). For comparison, cell surface AT_1 receptor protein levels were not significantly different between patients and controls, as determined in immunoblot (Figure 1F) and by binding assay (24.7 ± 4 fmol/mg protein, n = 8; and 22.8 ± 4.2 fmol/mg protein, n = 8; of patients and controls, respectively; data not shown). Altogether, the apparent molecular mass in SDS-PAGE and the determined AT_1 sequence point to a covalently bonded AT_1 receptor homodimer in patients.

Monocytic Factor XIIIA Transglutaminase

We analyzed the mechanism underlying the formation of covalently bonded AT_1 receptor homodimers to reveal its possible involvement in the enhancement of AT_1-dependent monocyte activation. The covalently dimerized AT_1 receptors were stable under reducing conditions of SDS-PAGE, excluding the possibility of disulfide bond formation. Receptor dimers can also be stabilized by crosslinking (AbdAlla et al., 2000), and transglutaminases mediate enzymatic crosslinking of membrane proteins by catalyzing the formation of stable amide bonds between proteins (Dutton and Singer, 1975). The predominant transglutaminase of monocytes is factor XIIIA (Muszbek et al., 1988). We determined monocytic factor XIIIA in immunoblot by factor XIIIA-specific antibodies (Figures 2A and 2B). Monocytic factor XIIIA protein levels of hypertensive patients were moderately (~1.6-fold) increased (Figure 2B). In agreement with previous findings (Muszbek et al., 1988), the intracellular factor XIIIA was not activated by thrombin cleavage, because the electrophoretic mobility of the thrombin-cleaved factor XIIIA was clearly distinguished from the intact factor XIIIA of monocytes (Figure 2C). Nevertheless, monocytic factor XIIIA of the patients displayed a markedly increased (3- to 4-fold) basal transglutaminase activity compared to that of the control individuals (Figure 2D). For comparison, the monocytic factor XIIIA activity after thrombin cleavage was increased only ~1.6-fold, confirming immunoblot results (Figure 2E versus Figure 2B). Thus, hypertensive patients displayed mark-

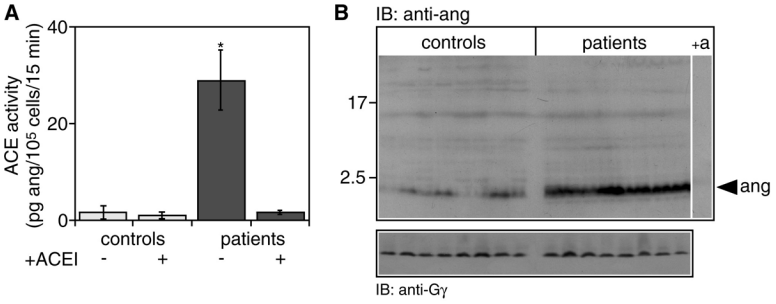

Figure 3. Angiotensin II Generation of Monocytes

(A) Monocytic ACE activity of the eight hypertensive patients and of the eight control individuals, as determined by the conversion of angiotensin I into angiotensin II and quantification of angiotensin II (ang) by radioimmunoassay. The ACE inhibitor captopril (+ACEI, 20 μM) suppressed monocytic angiotensin II generation (*$p < 0.01$ versus controls).

(B) Monocytic angiotensin II of the eight patients and of the eight controls as determined in immunoblot with affinity-purified angiotensin II-specific (anti-ang) antibodies. The protein amount was controlled by probing with anti-Gγ. The lane marked with +a is a specificity control performed in the presence of the antigen used for immunization.

edly increased monocytic factor XIIIA activity and a modest rise of factor XIIIA protein levels.

Mechanism of AT_1 Crosslinking

Does activated factor XIIIA account for the appearance of crosslinked AT_1 receptor dimers in humans? We activated the intracellular factor XIIIA of monocytes from a healthy individual by a calcium ionophore (Muszbek et al., 1995). While the ionophore increased basal transglutaminase activity (Figure 2F versus Figure 2D), the sole activation of factor XIIIA was not sufficient to induce crosslinking of AT_1 receptors (Figure 2G, lane 2 versus lane 1). We previously reported that angiotensin II induces formation of noncovalently bound AT_1 receptor homodimers, which can be stabilized by the addition of a synthetic crosslinker (AbdAlla et al., 2001b). The application of angiotensin II did not significantly affect factor XIIIA activity (Figure 2F) and did not induce the appearance of crosslinked AT_1 dimers (Figure 2G, lane 3). In contrast, the concomitant application of ionophore and angiotensin II induced the formation of crosslinked AT_1 dimers in a concentration-dependent manner (Figure 2G, lanes 4–6). The formation of crosslinked AT_1 dimers was suppressed either by application of the AT_1-specific antagonist losartan (Figure 2G, lane 7 versus lane 8) or by the transglutaminase inhibitors monodansyl cadaverine and cystamine (Figure 2G, lanes 10 and 11 versus lanes 9 and 12). These findings suggest that AT_1 receptor activation and (factor XIIIA) transglutaminase activity are required for AT_1 receptor dimerization and crosslinking.

To further analyze whether factor XIIIA crosslinks monocytic AT_1 receptors, we compared monocytes of factor XIIIA-deficient patients with those of healthy age-matched controls. Under basal conditions, the monocytic AT_1 receptor of factor XIIIA-deficient patients and of control individuals was not different, appearing in both groups as a monomer (data not shown). However, monocytes of the factor XIIIA-deficent patients showed also a monomeric AT_1 band upon application of the factor XIIIA activating agent ionomycin and of angiotensin II, whereas monocytes of the healthy individuals displayed high levels of crosslinked AT_1 dimers (Figure 2H). Accordingly, the monocytic factor XIIIA protein was only detectable in monocytes of the healthy individuals but was absent in factor XIIIA-deficient patients (Figure 2I). Together, these findings indicate that activated factor XIIIA is the predominant transglutaminase of monocytes, which crosslinks AT_1 receptors following angiotensin II stimulation.

Angiotensin II Generation of Monocytes

The formation of crosslinked AT_1 dimers requires activated factor XIIIA and angiotensin II. Searching for the source of angiotensin II, we determined angiotensin II plasma levels. In line with previous observations (Duggan et al., 1992), plasma levels of intact angiotensin II were not significantly different between patients and controls (7.2 ± 4.6 pM, $n = 8$; and 6.8 ± 2.8 pM, $n = 8$; respectively). In contrast, monocytes of hypertensive patients generated increased levels of angiotensin II from the precursor angiotensin I (Figure 3A). The monocytic angiotensin-converting enyzme (ACE) seemed responsible for the enhanced angiotensin II formation, because the ACE inhibitor captopril markedly suppressed the production of angiotensin II (Figure 3A). Increased ACE activity may also account for an increased local production of angiotensin II in vivo, because elevated levels of immunoreactive angiotensin II were also present in/on monocytes freshly isolated from patients (Figure 3B, upper panel; the lower panel shows equal protein loading by detecting Gγ). Thus, there is an increased ACE-dependent angiotensin II production and storage by monocytes of hypertensive patients.

Crosslinking of AT_1 Dimers in HEK Cells and In Vitro

Factor XIIIA and angiotensin II mediate the formation of crosslinked AT_1 receptors in monocytes. We next assessed the formation of crosslinked AT_1 dimers in AT_1-transfected HEK cells without or with coexpression of factor XIIIA (Figures 4A and 4B). Angiotensin II induced the appearance of crosslinked AT_1 dimers in a concentration-dependent manner of factor XIIIA-expressing cells preactivated with ionomycin (Figure 4B). Moreover, purified and preactivated factor XIIIA was capable to crosslink enriched angiotensin II-induced AT_1 dimers in vitro similarly to the synthetic crosslinker DST (Figure 4C, lanes 1–3), while a catalytic inactive factor $XIIIA^{C314A}$ mutant had no effect (Figure 4C, lanes 4 and 5). In contrast, coexpressed tissue transglutaminase (TG2) (Figure 4D, lanes 2 and 3 versus lane 1) preactivated with ionomycin did not efficiently stabilize angiotensin II-induced AT_1 dimers in HEK cells (Figure 4D, lanes 4–6).

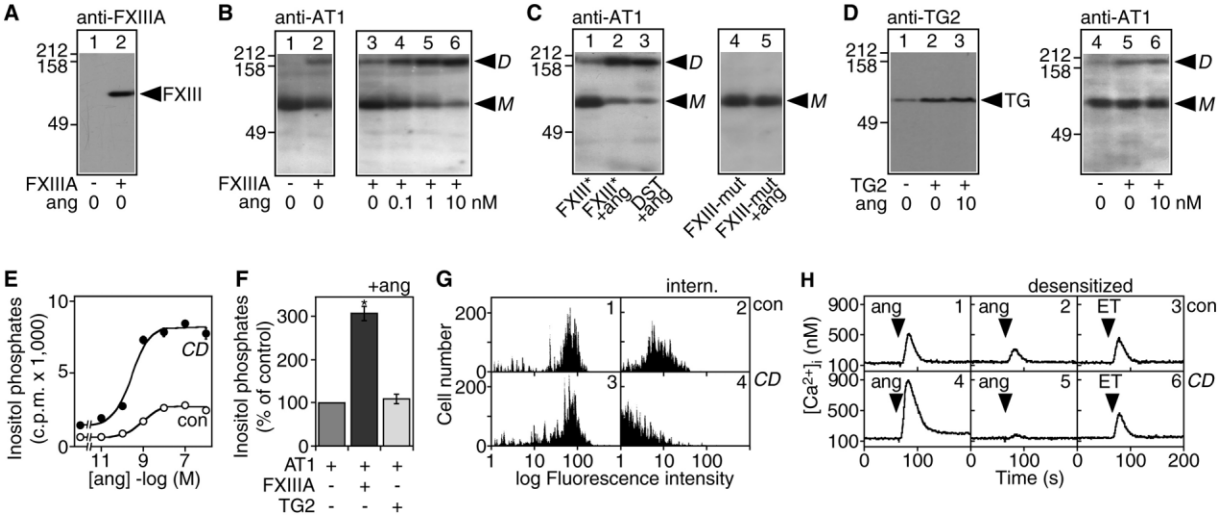

Figure 4. Enhanced Signaling and Desensitization of Crosslinked AT_1 Dimers

(A) Immunoblot detection of factor XIIIA (anti-FXIIIA) in mock-transfected control cells (lane 1, −) and in HEK cells expressing factor XIIIA (lane 2, +FXIIIA).

(B) Expression of factor XIIIA in HEK cells and activation by 0.1 μM ionomycin for 8 hr did not efficiently trigger AT_1 receptor dimerization compared to cells expressing AT_1 in the absence of factor XIIIA (lane 2 versus lane 1). Increasing concentrations of angiotensin II (8 hr) induced the appearance of crosslinked AT_1 dimers in cells expressing AT_1 and ionomycin-preactivated factor XIIIA (lanes 3–6).

(C) In vitro crosslinking of enriched AT_1 receptors by purified and preactivated factor XIIIA (FXIIIA*) in the absence (lane 1) or presence (lane 2) of 10 nM angiotensin II (ang). For comparison, angiotensin II-induced AT_1 dimers were crosslinked with DST (lane 3). As a control, the catalytically inactive $FXIIIA^{C315A}$ mutant (FXIII-mut) had no effect either in the absence or presence of angiotensin II (lanes 4 and 5).

(D) Immunoblot detection of transglutaminase 2 (TG) in TG2-transfected HEK cells (lanes 2 and 3) compared to mock-transfected cells (lane 1). Expression and activation (0.1 μM ionomycin, 8 hr) of TG2 did not efficiently crosslink AT_1 dimers upon angiotensin II (ang, 8 hr) stimulation (lanes 4–6).

(E) Inositol phosphate levels of HEK cells expressing crosslinked AT_1 dimers (*CD*) compared to control cells expressing noncrosslinked AT_1 (con). HEK cells expressing factor XIIIA and AT_1 receptors were pretreated either without (con) or with 0.1 μM ionomycin and 10 nM angiotensin II for 8 hr (*CD*), loaded with [^{3}H]inositol, washed, and stimulated for 20 min with increasing concentrations of angiotensin II as indicated; total inositol phosphates were extracted and quantified and are presented as c.p.m. Data are the mean ± SEM, n = 6.

(F) Angiotensin II-stimulated (10 nM) inositol phosphate levels of HEK cells expressing AT_1, and FXIIIA or TG2 as indicated. Cells were preincubated with ionomycin and angiotensin II (8 hr) as in (E). Data are expressed as % of control, i.e., the angiotensin II-stimulated increase in inositol phosphate levels of AT_1-expressing cells (100%). Data are the mean ± SEM, n = 6; *$p < 0.01$.

(G) Determination of cell surface AT_1 receptors of HEK cells expressing noncrosslinked AT_1 receptors (panels 1 and 2; con) or crosslinked AT_1 dimers (panels 3 and 4; *CD*). Crosslinked AT_1 dimers were induced as in (E), and cell surface AT_1 receptors were quantified by FACS using AT_1-specific antibodies. Cells were treated without (panels 1 and 3) or with (panels 2 and 4) 100 nM angiotensin II for 30 min at 37°C (intern.). Angiotensin II led to a moderate decrease in the fluorescence intensity of cells expressing noncrosslinked AT_1 receptors (panel 2) while markedly decreasing that of cells expressing crosslinked AT_1 dimers (panel 4) indicative of enhanced receptor internalization of crosslinked AT_1 dimers.

(H) Angiotensin II- and endothelin-stimulated increase in $[Ca^{2+}]_i$ of HEK cells grown on glass cover slips. Cells expressed factor XIIIA and noncrosslinked AT_1 (con, panels 1–3) or crosslinked AT_1 dimers induced as in (E) (*CD*, panels 4–6). Cells were washed, loaded with fura-2, and stimulated as indicated with 10 nM angiotensin II (ang, panels 1, 2, 4, and 5) or endothelin-1 (ET, panels 3 and 6) without (panels 1 and 4) or with prior treatment with 100 nM angiotensin II for 5 min at 37°C (followed by washing) to induce AT_1 desensitization (desensitized, panels 2, 3, 5, and 6).Single experiments are representative of three independent experiments, each with similar results.

This finding may either reflect that AT_1 is a poor substrate of TG2 due to different substrate specificities of factor XIIIA and TG2 (Gorman and Folk, 1980), or that receptor-stimulated GTP loading inhibited TG2 activity in cells (Zhang et al., 1998). Altogether, factor XIIIA crosslinks AT_1 receptor dimers in transfected HEK cells, in native monocytes, and in vitro.

Enhanced Signaling and Desensitization of Crosslinked AT_1 Dimers

We measured the phospholipase C-dependent increase in cellular inositol phosphate levels predominantly mediated by the AT_1-stimulated activation of $G\alpha_{q/11}$. In this assay, crosslinked AT_1 dimers produced almost 3-fold increased cellular inositol phosphate levels compared to noncrosslinked AT_1 receptors upon stimulation with different concentrations of angiotensin II (Figure 4E). Moreover, expression and ionomycin activation of TG2 did not lead to significant enhancement of angiotensin II-stimulated inositol phosphate signaling (Figure 4F), consistent with the result of Figure 4D. Increased signaling of the crosslinked AT_1 dimers was accompanied by an enhanced $G\alpha_{q/11}$ activation, as determined by the AT_1 receptor-stimulated GTPγS binding to $G\alpha_q$ (cf. Figure 5C).

The amount of expressed AT_1 receptors in transfected HEK cells was controlled by fluorescence-activated cell sorting (FACS) analysis using AT_1-specific antibodies. The analysis revealed similar staining of cells expressing noncrosslinked AT_1 receptors and crosslinked AT_1 di-

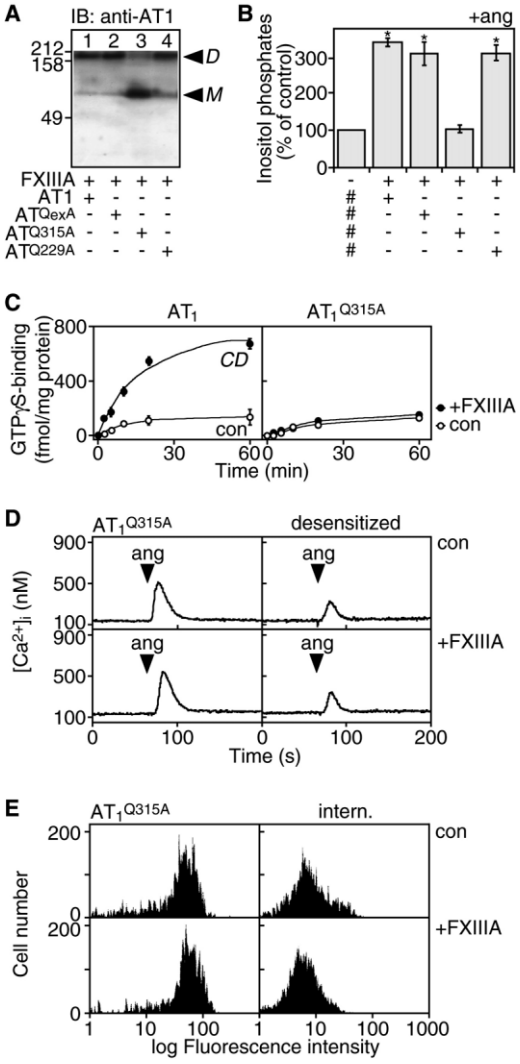

Figure 5. Glutamine^{315} of the AT_1 Receptor Is a Target of Factor XIIIA

(A) Mutated AT_1^{Q315A} receptor is not a target of factor XIIIA, while wild-type AT_1, $AT_1^{Q15/257/267A}$ (AT_1^{QexA}), and AT_1^{Q229A} form crosslinked dimers as determined in immunoblot of angiotensin II-stimulated HEK cells expressing the indicated AT_1 receptor and ionomycin-preactivated factor XIIIA.

(B) Ionomycin-preactivated intracellular factor XIIIA did not enhance the angiotensin II-stimulated (10 nM) inositol phosphate signal mediated by AT_1^{Q315A}, which is not a target of factor XIIIA-mediated crosslinking, while factor XIIIA-dependent crosslinking of wild-type AT_1, of AT_1^{QexA}, and of AT_1^{Q229A} enhanced AT_1-stimulated signaling compared to similarly pretreated control cells expressing the indicated receptor in the absence of factor XIIIA (column 1, #, 100%). Data are the mean ± SEM, n = 6; *$p < 0.01$.

(C) Factor XIIIA-crosslinked AT_1 dimers (*CD*) stimulate enhanced GTPγS binding to $G\alpha_q$ as determined with membranes (20 μg/point) of angiotensin/ionomycin-pretreated (8 hr) HEK cells expressing AT_1 (left panel) or AT_1^{Q315A} (right panel) and FXIIIA as indicated. The specific angiotensin II-stimulated (100 nM) GTPγS binding to $G\alpha_q$ is presented. Receptor expression levels (~100 fmol/mg protein), FXIIIA and $G\alpha_q$ levels—as applicable—were comparable. Data are the mean ± SD, n = 6.

(D) The angiotensin II-stimulated calcium transient of fura-2-loaded and angiotensin/ionomycin-pretreated (8 hr) HEK cells expressing AT_1^{Q315A} and factor XIIIA as indicated. Desensitization of AT_1^{Q315A} was induced by angiotensin II (100 nM; right panels).

(E) Receptor internalization of AT_1^{Q315A} is not affected by factor XIIIA. Cell surface AT_1^{Q315A} receptors were determined by FACS analysis without (left panels) or with (right panels) pretreatment with angiotensin II to induce receptor internalization.

mers (Figure 4G, panels 1 and 3). Application of a saturating concentration of angiotensin II triggered AT_1 receptor internalization, as reflected by a decrease in cell surface fluorescence intensity (Figure 4G). Internalization of the crosslinked AT_1 dimers was significantly increased compared to the noncrosslinked AT_1 receptors (Figure 4G, panel 4 versus panel 2).

The $G\alpha_{q/11}$-dependent signal transduction of the crosslinked AT_1 dimers was further analyzed by measuring the angiotensin II-stimulated transient rise in intracellular calcium, $[Ca^{2+}]_i$. In agreement with Figure 4E, the crosslinked AT_1 dimers displayed markedly increased signaling compared to noncrosslinked AT_1 receptors (Figure 4H, panel 4 versus panel 1). Receptor activation induces receptor desensitization, which is visible by a decreased receptor-stimulated response. Angiotensin II-prestimulated cells revealed partial desensitization of noncrosslinked AT_1 receptors in contrast to full desensitization of crosslinked AT_1 dimers as measured by the angiotensin II-stimulated calcium transient (Figure 4H, panel 5 versus panel 2). As a control, cells expressing either noncrosslinked or crosslinked AT_1 receptors were equally responsive to endothelin-1 with or without prior desensitization of AT_1 (Figure 4H, panels 3 and 6; and data not shown). Thus, crosslinked AT_1 dimers display enhanced $G\alpha_{q/11}$-stimulated signaling, increased internalization, and desensitization.

Glutamine^{315} in the Cytoplasmic Side of AT_1 Is a Target of Factor XIIIA

Transglutaminases catalyze the displacement of amide ammonia at the γ position in glutamine by replacing it with an ε amino group from a suitable lysine. An amine acceptor glutamine of the AT_1 receptor for factor XIII-mediated crosslinking was determined by site-directed mutagenesis. While removal of the extracellularly located glutamines in AT_1 did not affect the formation of crosslinked AT_1 receptor dimers (Figure 5A, lane 2 versus lane 1), exchange of glutamine^{315} significantly decreased the amount of crosslinked AT_1 dimers formed upon angiotensin II stimulation in the presence of activated factor XIIIA (Figure 5A, lane 3). As a control, AT_1^{Q315A} was capable to form homodimers upon angiotensin II stimulation, as assessed in immunoblot after stabilization of AT_1^{Q315A} dimers with the synthetic crosslinker DST (data not shown). As an additional control, removal of glutamine^{229} also did not markedly alter AT_1 receptor dimerization (Figure 5A, lane 4). Together, these findings suggest that glutamine^{315} in the cytoplasmic tail of the AT_1 receptor is a target of factor XIII-mediated crosslinking.

Activated factor XIIIA led to enhanced signaling of the wild-type AT_1 receptor and of the AT_1 receptor mutants, which were targets of factor XIIIA (Figure 5B). In contrast, activated factor XIIIA did not affect signaling of the AT_1^{Q315A} mutant that was not crosslinked by factor XIIIA (Figure 5B). In line with this finding, the receptor-stimulated $G\alpha_q$ activation mediated by the crosslinked wild-type AT_1 dimers was significantly enhanced compared

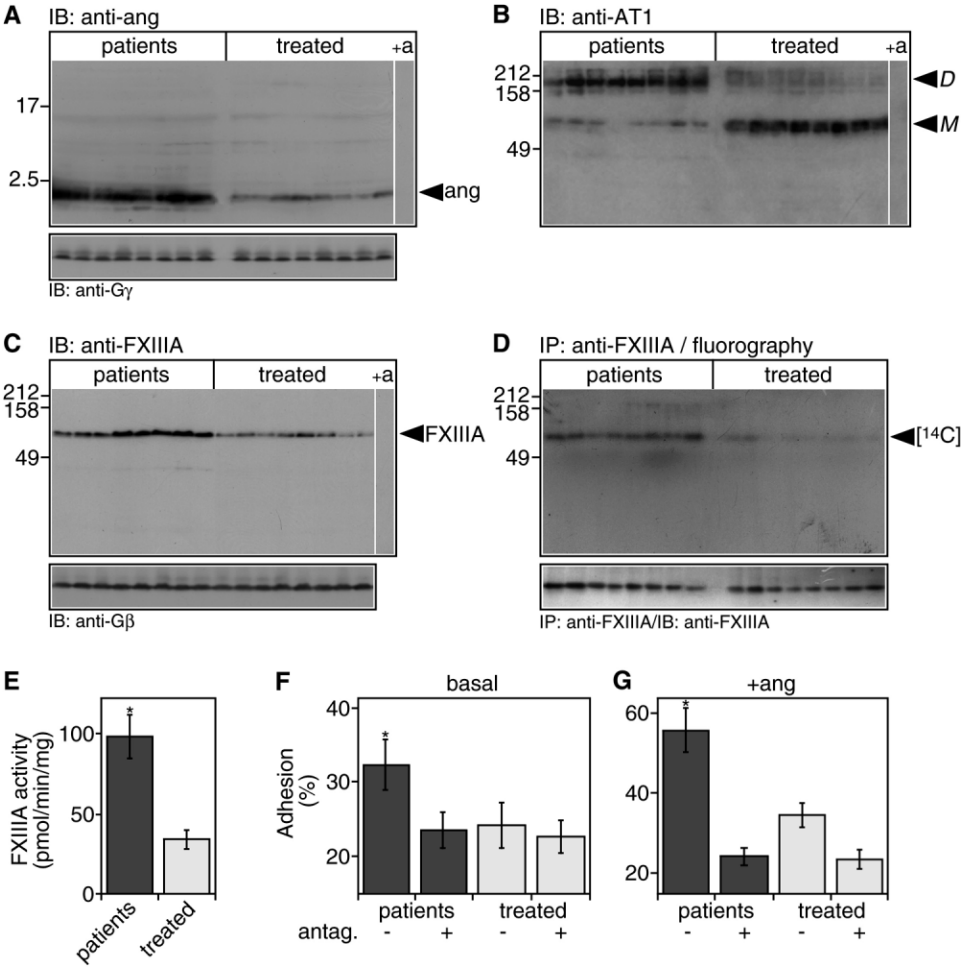

Figure 6. ACE Inhibitor Therapy of Hypertensive Patients Normalizes the Monocytic AT_1 System and Covalent AT_1 Dimerization

(A) Immunoblot detection of monocytic angiotensin II of eight hypertensive patients before (patients) and after 3 months of ACE inhibitor treatment (treated).

(B) Monocytic AT_1 receptor detection in immunoblot with affinity-purified AT_1-specific antibodies of eight patients before (patients) and after (treated) ACE inhibitor treatment.

(C) ACE inhibitor treatment decreased elevated monocytic factor XIIIA levels of the eight patients as determined in immunoblot. Lanes marked with +a (A–C) are specificity controls with immunoblots performed in the presence of the corresponding antigens used for immunization. The protein amount was controlled in the lower panels by probing with anti-Gγ (A) or anti-Gβ (C).

(D) Basal monocytic factor XIIIA activity of patients before and after ACE inhibitor treatment as assessed by active-site SH-labeling with [^{14}C]iodoacetamide followed by immunoprecipitation (IP) with affinity-purified factor XIIIA-specific antibodies and fluorography (upper panel). The lower panel controls immunoprecipitated monocytic factor XIIIA (IP) by factor XIIIA-specific antibodies in immunoblot (IB).

(E) Basal monocytic factor XIIIA transglutaminase activity of patients before and after ACE-inhibitor therapy as determined by [^{3}H]putrescine incorporation.

(F and G) Basal and angiotensin II (+ang)-stimulated monocyte adhesiveness of patients before and after ACE-inhibitor therapy. Monocytes were pretreated with 5 μM losartan (± antag.) as indicated (*$p < 0.02$ versus treated).

to noncrosslinked AT_1 receptors, but GTPγS binding to $G\alpha_q$ mediated by the AT_1^{Q315A} receptor mutant was not significantly enhanced under similar conditions (Figure 5C). Likewise, factor XIIIA did not affect calcium signaling and internalization of the AT_1^{Q315A} receptor mutant (Figures 5D and 5E). Together, these findings further confirm that crosslinking of AT_1 receptor dimers leads to enhanced AT_1 receptor-stimulated G protein activation, signaling, and desensitization.

ACE Inhibition Normalizes the Monocytic AT_1 Receptor System

The interplay between activated factor XIIIA and angiotensin II is essential for the production of hyperresponsive crosslinked AT_1 dimers of monocytes. To explore the importance of this relationship in vivo, we analyzed monocytes of hypertensive patients before and after 3 months of an ACE inhibitor-based antihypertensive therapy. ACE inhibition significantly decreased the elevated monocytic angiotensin II levels (Figure 6A) and reduced the pathologically elevated levels of crosslinked AT_1 dimers (Figure 6B). Concomitantly, factor XIIIA protein levels and basal factor XIIIA activity were decreased as determined in immunoblot or by active-site SH labeling and by [^{3}H]putrescine incorporation, respectively (Figures 6C–6E). Moreover, the AT_1-dependent monocyte adhesion of patients normalized upon ACE inhibition (Figures 6F and 6G). Together, these find-

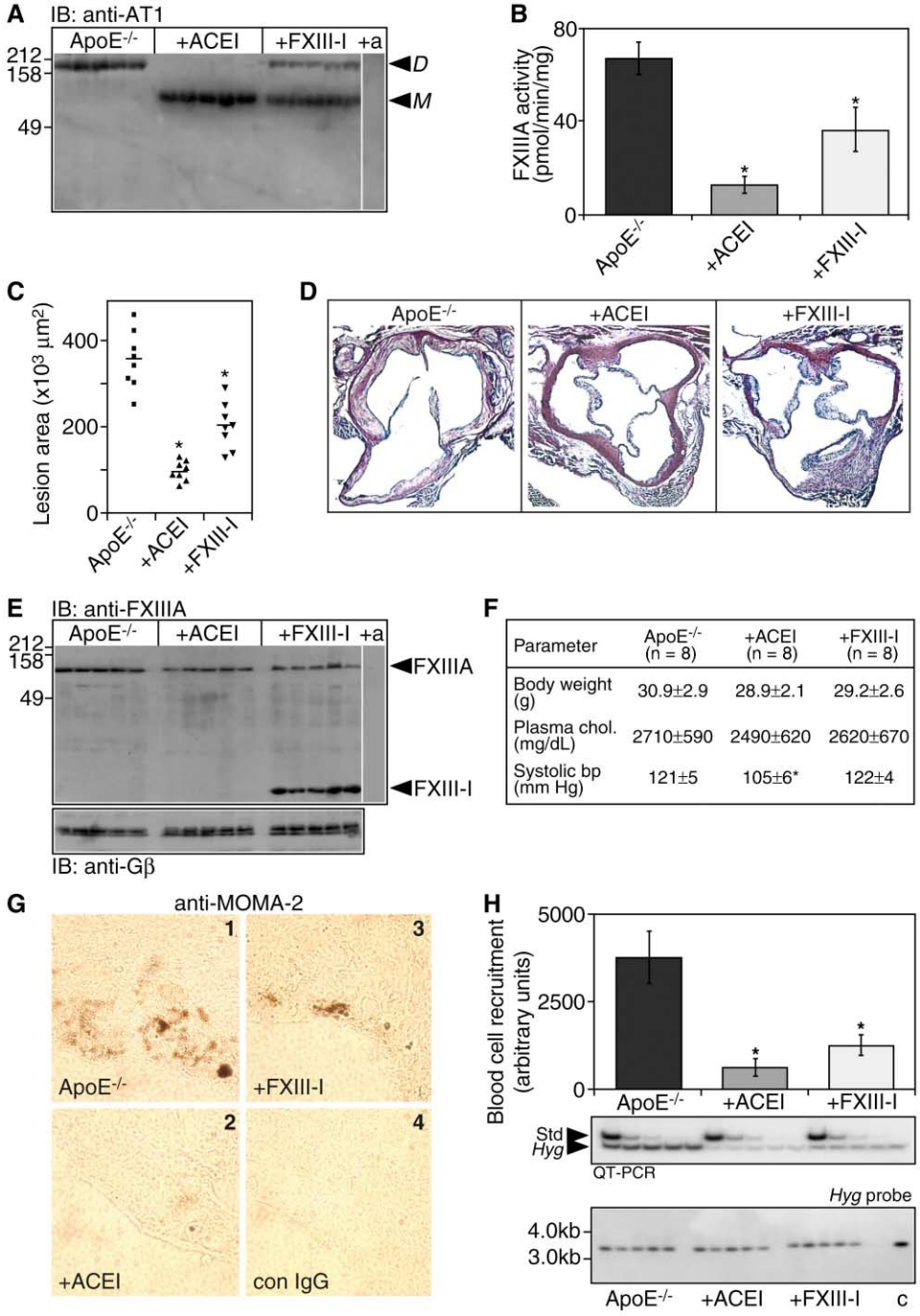

Figure 7. Crosslinked AT_1 Dimers in ApoE-Deficient Mice

(A) Treatment of MSCV-transduced ApoE$^{-/-}$ control mice (ApoE$^{-/-}$) with ACE inhibitor captopril (+ACEI) or retrovirus-mediated expression of a factor XIIIA inhibitor (+FXIII-I) decreased the amount of crosslinked AT_1 dimers of monocytes from 26-week-old mice.

(B) Basal factor XIIIA activity of monocytes from 26-week-old MSCV-transduced control (ApoE$^{-/-}$), ACE inhibitor-treated (+ACEI), or factor XIIIA inhibitor-expressing (+FXIII-I) ApoE$^{-/-}$ mice (n = 8).

(C and D) Quantification (C) of aortic root lesion area of 26-week-old MSCV-transduced control (ApoE$^{-/-}$), ACE inhibitor-treated (+ACEI), or factor XIIIA inhibitor-expressing (+FXIII-I) ApoE$^{-/-}$ mice (n = 8). Representative hematoxylin-eosin-stained sections (original magnification ×50) representing the mean lesion area of each group (D).

(E) Immunoblot of factor XIIIA (FXIIIA) and of the factor XIIIa inhibitor (N^{73}–D^{98}; FXIII-I) of monocytes from 26-week-old control (ApoE$^{-/-}$), ACE inhibitor-treated (+ACEI), or MSCV-FXIII-I-transduced (+FXIII-I) ApoE$^{-/-}$ mice.

(F) Body weight, plasma cholesterol, and systolic blood pressure of the three groups.

(G) Immunohistochemical staining of monocytes/macrophages in aortic valve sections of 16-week-old MSCV-transduced control (ApoE$^{-/-}$; panel 1), ACE inhibitor-treated (+ACEI; panel 2), or MSCV-FXIII-I-transduced (+FXIII-I; panel 3) ApoE$^{-/-}$ mice with MOMA-2-specific antibodies following antigen retrieval (original magnification ×600). Panel 4 shows a section of ApoE$^{-/-}$ mice stained with control IgG. A representative section is shown for each group.

ings strongly suggest that ACE-dependent angiotensin II generation is causally involved in the formation of factor XIIIA-crosslinked AT_1 receptor dimers on monocytes of hypertensive patients. Moreover, the appearance of the crosslinked AT_1 dimers may contribute to an enhanced AT_1-dependent monocyte adhesiveness.

Inhibition of ACE or of Intracellular Factor XIIIA of Blood Cells Suppresses AT_1 Receptor Dimerization and Symptoms of Atherosclerosis in ApoE-Deficient Mice

Since enhanced monocyte adhesion is a key feature of atherogenesis, we assessed the AT_1 system of hypercholesterolemic ApoE-deficient ($ApoE^{-/-}$) mice, which serve as a model of atherosclerosis. Monocytes of $ApoE^{-/-}$ mice displayed increased levels of crosslinked AT_1 receptor dimers at 6 weeks of age (data not shown) and at the end of the observation period at 26 weeks (Figure 7A). Treatment for 10 weeks with the ACE inhibitor captopril significantly decreased the amount of crosslinked AT_1 dimers on monocytes and suppressed the monocytic factor XIIIA activity compared to untreated $ApoE^{-/-}$ mice (Figures 7A and 7B). In agreement with the antiatherosclerotic effect of ACE inhibitors in this model (Hayek et al., 1998), the mean lesion area of the aortic root was substantially reduced upon ACE inhibition (Figures 7C and 7D).

To assess the involvement of factor XIIIA, we inhibited the intracellular factor XIIIA activity of blood cells in vivo by retroviral transduction of a factor XIIIA inhibitor (Achyuthan et al., 1993). The expression of the factor XIIIA inhibitor was demonstrated in immunoblots of bone marrow cells, of macrophages, and of circulating monocytes of bone marrow-transplanted $ApoE^{-/-}$ mice transduced with the factor XIIIA inhibitor-encoding retrovirus (data not shown and Figure 7E). Expression of the factor XIII inhibitor in $ApoE^{-/-}$ mice was detected as early as 3 weeks after bone marrow transplantation and persisted until the end of the observation period at 26 weeks (data not shown). The factor XIIIA inhibitor significantly decreased the levels of crosslinked AT_1 dimers and suppressed the factor XIIIA activity of monocytes (Figures 7A and 7B). Concomitantly, the atherosclerotic lesion area was significantly reduced (Figures 7C and 7D). Captopril or the factor XIIIA inhibitor did not affect plasma cholesterol level or body weight, while systolic blood pressure was decreased by captopril (Figure 7F).

In agreement with the enhanced AT_1-dependent monocyte adhesiveness of atherosclerosis-prone patients (cf. Figures 1 and 6), monocyte/macrophage infiltration of the aorta of 16-week-old $ApoE^{-/-}$ mice was also significantly decreased upon ACE or factor XIIIA inhibition, as determined by immunostaining of monocytes/macrophages in aortic root sections with MOMA-2-specific antibodies (Figure 7G). Quantitative PCR tracking the retrovirus-derived hygromycin phosphotransferase (*Hyg*) gene in the aortic DNA confirmed the decrease in the recruitment of blood cells into the aorta of ACE- or factor XIII inhibitor-treated mice (Figure 7H, upper and middle panels). As a control, Southern blot analysis of the genomic DNA of circulating blood cells from the three treatment groups revealed comparable provirus integration of MSCV or MSCV-FXIII-I (Figure 7H, lower panel). Concomitantly to the suppression of blood cell infiltration of the aorta, mean aortic root lesion area was also significantly reduced upon ACE or factor XIIIA inhibition (63,480 $\pm$ 10,930 μm^2, 17,480 $\pm$ 3,650 μm^2, and 34,670 $\pm$ 7,530 μm^2 of control, ACE or factor XIIIA inhibitor-treated 16-week-old $ApoE^{-/-}$ mice, respectively; $n = 8$; $\pm$ SD; $p < 0.01$). Thus, angiotensin II-triggered and factor XIIIA-crosslinked AT_1 dimers of circulating blood cells are related to the appearance of symptoms of atherosclerosis in this experimental model.

Discussion

We herein identify hyperresponsive crosslinked angiotensin II-induced AT_1 receptor homodimers, which are covalently bonded by the transglutaminase activity of intracellular factor XIIIA. This previously unrecognized species of crosslinked receptor homodimers is distinguished from dissociable constitutive receptor clusters/oligomers/dimers or monomers by covalent linkage of two receptor molecules. Considering the finding that two receptors could interact with one G protein (Baneres and Parello, 2003), a unique nondissociable entity of two crosslinked AT_1 receptors may provide a kinetically favorable interface for interaction with the G protein compared to two individual receptors or to a dissociable receptor complex, thereby accounting for enhanced activation and signaling.

Increased levels of hyperresponsive crosslinked AT_1 dimers were present on monocytes of patients with the atherogenic risk factor hypertension and correlated with an increased angiotensin II-dependent monocyte activation and adhesiveness. An enhanced monocyte adherence to the endothelium at sites predisposed to atherosclerosis is one of the earliest detectable cellular responses in atherogenesis leading to the formation of atherosclerotic lesions (Faggioto et al., 1984; Gerrity et al., 1985). Monocyte recruitment into the artery involves not only an alteration of the arterial wall but also requires functional changes in the circulating monocyte, with a mutual exchange of activating stimuli between monocytes and endothelium (Gerrity et al., 1985). In this context, the present work may delineate a mechanism accounting for functional changes of monocytes at the

(H) Quantitative assessement of blood cell recruitment into the aorta of 16-week-old MSCV-transduced control ($ApoE^{-/-}$), MSCV-transduced ACE inhibitor-treated (+ACEI), or MSCV-FXIII-I-transduced (+FXIII-I) $ApoE^{-/-}$ mice measured by quantitative PCR (QT-PCR) amplifying the hygromycin phosphotransferase gene of the transduced MSCV (arbitrary units, upper panel, $n = 8$). Representative autoradiography of the PCR products obtained from serial dilutions of the internal standard (Std, 220 bp) and a constant amount of aortic DNA (*Hyg*, 180 bp) from an individual mouse of each group (middle panel). Southern blot of KpnI-digested genomic DNA from circulating blood cells of five mice of each group hybridized with a *Hyg* probe shows fragments of $\sim$3.3 and 3.4 kb indicative of comparable levels of MSCV or MSCV-FXIII-I proviruses (lower panel). Data are the mean $\pm$ SD, *$p < 0.01$.

onset of atherogenesis, which is based on the appearance of hyperactive crosslinked AT_1 receptor dimers. In line with such a hypothesis is the finding that high levels of crosslinked AT_1 dimers were present on monocytes of hypercholesterolemic ApoE-deficient mice, and inhibition of angiotensin II generation or of intracellular factor XIIIA activity suppressed the appearance of crosslinked AT_1 receptor dimers and symptoms of atherosclerosis.

Formation of crosslinked AT_1 dimers requires the activation of the angiotensin II-AT_1 system and of factor XIIIA. A wealth of data demonstrated the increased activity of local ACE-dependent angiotensin II-generating systems in hypertension and vascular disease (Dzau, 2001), and pressure as well as shear stress or inflammatory stimuli directly induces the expression of ACE (Gosgnach et al., 2000; Lazarus et al., 1994; Schunkert et al., 1990). In agreement with these observations, monocytic ACE activity and/or angiotensin II generation were increased in patients with the atherogenic risk factor hypertension and in hypercholesterolemic ApoE-deficient mice. Thus, activation of the monocytic angiotensin II-AT_1 system may be triggered by vascular inflammation, which develops as a consequence of persisting atherogenic risk factors such as hypertension or hypercholesterolemia. In addition to activation-dependent receptor dimerization, constitutive receptor clustering and dimerization have been reported (Fotiadis et al., 2004), and the concept of a dynamic interplay between receptor clustering and ligand-induced receptor rearrangement is emerging (Franco et al., 2003). However, the amount of crosslinked AT_1 dimers was negligible in vivo in the absence of angiotensin II. This finding could reflect (1) that constitutive AT_1 receptor clusters/dimers are not abundant in vivo, and/or (2) that structural differences between constitutive receptor clusters/dimers and agonist-induced dimers preclude covalent modification by factor XIIIA.

Besides enhancing the release of angiotensin II, atherogenic risk factors may also induce factor XIIIA (Ma and Liew, 2003). On the other hand, the sole accumulation of factor XIIIA protein is not necessarily associated with its crosslinking activity (Zhang et al., 1998). The increased factor XIIIA activity may therefore be the major event contributing to crosslinking of AT_1 dimers. Activation of intracellular factor XIIIA can be mediated by an altered ion homeostasis (Polgár et al., 1990) and/or by oxidative stress. Increased sodium and calcium levels are characteristic features of hypertension and vascular disease (Caimi et al., 1997; Postnov and Orlov, 1985), and the atherosclerosis-promoting effect of oxidative stress is well known. Thus, crosslinked AT_1 receptor dimers of monocytes may reflect the concerted action of a panoply of different atherogenic stimuli. In line with this conclusion is the finding that ACE inhibitor treatment of atherosclerotic mice suppressed the development of atherosclerosis and concomitantly normalized factor XIIIA activity. Due to the long half-life of >5 days, the crosslinked AT_1 dimers seem to be barely affected by short-term changes of medication, diet, or lifestyle. In view of these data, it seems appropriate to propose that crosslinked AT_1 dimers sustain the process of atherogenesis by chronic sensitization of circulating monocytes.

Experimental Procedures

Cell Culture and Functional Assays

HEK cells were cultured and transfected with the indicated cDNAs; mutants were generated by PCR; and cellular inositol phosphate levels, $[Ca^{2+}]_i$, and the AT_1 receptor-stimulated increase in the GTPγS binding of $G\alpha_q$ were determined as described (AbdAlla et al., 2000; Lorenz et al., 2003). All solutions and chemicals used in monocyte isolation and activation procedures were endotoxin-free (endotoxin <0.008 ng/ml). Human or mouse peripheral blood monocytes were isolated from heparin- or EDTA-anticoagulated blood diluted with PBS by density gradient centrifugation through Ficoll-Paque or Optiprep followed by plastic adherence, or by Nycoprep density gradients. Cell viability was $>98\%$, as determined by trypan blue exclusion and propidium iodide staining, and monocyte purity as determined by immunofluorescence staining of cell-specific antigens (CD14/CD45, CD3, CD19, CD68, and CD163) was $>80\%$. Monocyte release of interleukin-1β into culture supernatant was determined by ELISA (R&D Systems), transglutaminase activity was assessed in cell lysates by [^{3}H]putrescine incorporation assay (Muszbek et al., 1995), and adhesion of monocytes to endothelial cells was determined as described (Dörffel et al., 1999). The intraassay variations were less than 5%, and the day-to-day variation in one subject was less than 10%. Group data are expressed as means $\pm$ SD. Mean values were compared with the use of paired or unpaired Student's t tests, as appropriate. P values <0.05 were considered significant.

Fluorescence-Activated Cell Sorting

Cell surface AT_1 receptors of transfected HEK cells were determined by FACS using affinity-purified AT_1-specific antibodies raised against an antigen corresponding to the receptor's third extracellular loop, as described (Quitterer et al., 1999).

Protein Detection in Immunoblot and Protein Sequencing

AT_1 receptor monomers and dimers of monocytes and of HEK cells were determined in immunoblot with AT_1-specific antibodies as described (AbdAlla et al., 2000, 2001a, 2001b). The intraassay variation for AT_1 receptor detection and the day-to-day variation in one subject were less than 10%. Tissue levels of the factor XIIIA protein were determined in immunoblot with factor XIIIA-specific antibodies. Specificity and crossreactivity of the antibodies with human or mouse factor XIIIA were confirmed in immunoblot. Angiotensin II levels of monocytes were determined in immunoblot after Tricine-SDS-PAGE with angiotensin II-specific antibodies displaying minimal crossreactivity to angiotensin I ($<1\%$). As controls, protein levels of Gγ and Gβ proteins were determined with Gγ- or Gβ-specific antibodies.

Monocytes were isolated from hypertensive patients for amino-terminal protein sequencing of the crosslinked AT_1 dimer. After membrane preparation, the AT_1 receptor was solubilized and affinity purified by lectin- and immuno-affinity chromatography. Purified AT_1 receptors were desalted and concentrated using a Centricon filtration unit (exclusion limit 100,000 Da). Proteins were subjected to SDS-PAGE, transferred to a PVDF membrane, and analyzed on an ABI 494A Procise HT (IBA GmbH, Goettingen, Germany).

Patients

Monocytes were isolated from the peripheral blood of 16 patients diagnosed with essential hypertension (eight males, 53 $\pm$ 3 years, 74 $\pm$ 7 kg, total plasma cholesterol 176 $\pm$ 13 mg/dL, systolic/diastolic blood pressure of 178 $\pm$ 7/103 $\pm$ 5 mm Hg). Hypertensive patients responded to an ACE inhibitor-based antihypertensive therapy (captopril 12.5 mg three times daily titrated to 25–50 mg three times daily plus—if required—thiazide diuretic to reach a target blood pressure of $<140/90$ mm Hg), i.e., 3 months of antihypertensive therapy decreased blood pressure significantly (systolic/diastolic bp 134 $\pm$ 5/83 $\pm$ 5 mm Hg). Monocytes were analyzed before any drug administration and after 3 months of therapy. Eight healthy individuals served as controls matching the patients according to age, body weight, and gender (53 $\pm$ 4 years, 72 $\pm$ 7 kg, eight males, total plasma cholesterol 175 $\pm$ 12 mg/dL, systolic/diastolic bp 128 $\pm$ 6/78 $\pm$ 3 mm Hg). Patients (and controls) with other diseases or

abnormal laboratory parameters, elevated LDL levels, or atherosclerosis were excluded.

Monocytes were isolated from the peripheral blood drawn for routine diagnosis of five patients with congenital factor XIIIA deficiency and of six age-matched control individuals. Patients were on regular replacement therapy, and blood was drawn before factor XIIIA replacement. Residual factor XIIIA activity in plasma was 1%–5%, and factor XIIIA activity of platelets and monocytes was not detectable (<0.1%). Factor XIIIA deficiency was diagnosed by positive urea solubility test of fibrin clot and essentially no enzymatic activity of factor XIIIA (<1% of normal).

Informed consent was obtained from all patients and control individuals, and the study was approved by the ethics review committee at the University of Cairo.

ApoE-Deficient Mouse Model of Atherosclerosis

ApoE-deficient C57BL/6J mice (Taconic) were maintained on a standard pellet rodent chow. At 11–12 weeks of age, $ApoE^{-/-}$ mice were lethally irradiated and received MSCV-transduced bone marrow cells intravenously (Dinauer et al., 1999). A Western-type diet containing 21% fat and 0.15% cholesterol was initiated 4 weeks after bone marrow transplantation and was continued until the end of the observation period at 25–26 weeks. Retroviral transduction was performed essentially as described (Dinauer et al., 1999; Ishiguro et al., 2001). The retroviral vector MSCV-FXIII-I was generated by subcloning a PCR-generated cDNA fragment encoding the factor XIIIA transglutaminase inhibitor, factor $XIIIA^{N73\text{-}D98}$ (Achyuthan et al., 1993), and containing an initiation ATG and a Kozak consensus ribosome binding site into the MSCV vector backbone.

Recipient $ApoE^{-/-}$ mice were divided into three groups: one group was transplanted with MSCV-transduced cells, the second group was transplanted with MSCV-transduced cells and was given captopril (50 mg/kg/d) starting 4 weeks after bone marrow transplantation, and the third group was transplanted with MSCV-FXIII-I-transduced bone marrow cells. After 10 weeks of treatment (14 weeks post bmt), mice were sacrificed, blood was collected by cardiac puncture, and monocytes were prepared as described above. Atherosclerosis in $apoE^{-/-}$ mice was followed by quantitative aortic sinus measurement of hematoxylin-eosin-stained paraffin-embedded sections cut with an automated microtome (HM350 SV, Microm) as described (Cayatte et al., 2000; Paigen et al., 1987). For quantification of the lesions, five sections (10 μm) per mouse were measured, taken, at 80 μm intervals, of the aortic bulb from the commissures of the aortic leaflets and upward. Lesions were quantified by using a computerized image analysis system (Leica DMRA-HC microscope equipped with INTAS digital microscope imaging system) and SigmaScan Pro 5.0 software (SPSS). Lesion area was measured in a blinded manner. For each animal, the average lesion area of five sections is given. Transduction of $ApoE^{-/-}$ mice with control MSCV did not affect lesion area. Plasma cholesterol was determined using a standard enzymatic kit, and systolic blood pressure was measured by the tail-cuff method.

To assess the effect of factor XIIIA inhibitor expression on monocyte/macrophage infiltration of the aorta, viral transduction was performed on 6-week-old $ApoE^{-/-}$ mice, and after 10 weeks under regular chow diet (± captopril), recruitment of monocytes/macrophages into the aorta was determined by immunohistochemistry. Paraffin-embedded aortic root sections were immunostained for monocytes/macrophages using monoclonal anti-MOMA-2 antibodies (Serotec) followed by peroxidase-conjugated secondary antibodies and DAB-enhanced liquid substrate system detection (Sigma). For each animal, three sections were evaluated, and the area stained with DAB of the total crosssectional vessel wall area was determined. Concomitantly, recruitment of circulating blood cells into the aorta was quantified by quantitative PCR, tracking the presence of the transduced control MSCV (control or captopril group) or MSCV-FXIII-I in the aorta (Kim et al., 2000). To control for comparable provirus integration between the three treatment groups, the presence of the provirus in the genomic DNA of circulating blood cells was determined by Southern blot of KpnI-digested DNA hybridized with a hygromycin phosphotransferase DNA probe. Data were statistically analyzed by multiple ANOVA followed by post hoc analysis by either parametric or nonparametric tests, as appropriate for the specific data set.

All animal experiments were reviewed and approved by the committees on animal research at the Universities of Cairo and of Hamburg and were conducted in accordance with the NIH guidelines.

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