Unacylated Ghrelin Rescues Endothelial Progenitor Cell Function in Individuals With Type 2 Diabetes

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OBJECTIVE—Acylated ghrelin (AG) is a diabetogenic and orexigenic gastric polypeptide. These properties are not shared by the most abundant circulating form, which is unacylated (UAG). An altered UAG/AG profile together with an impairment of circulating endothelial progenitor cell (EPC) bioavailability were found in diabetes. Based on previous evidence for the beneficial cardiovascular effects of AG and UAG, we investigated their potential to revert diabetes-associated defects.

RESEARCH DESIGN AND METHODS—Healthy human subjects, individuals with type 2 diabetes, and *ob/ob* mice were AG or UAG infused. EPC mobilization in patients and mice was evaluated, and the underlying molecular mechanisms were investigated in bone marrow stromal cells. Recovered EPCs were also evaluated for the activity of senescence regulatory pathways and for NADPH oxidase activation by knocking down $p47^{phox}$ and Rac1. Finally, UAG modulation of human EPC vasculogenic potential was investigated in an in vivo mouse model.

RESULTS—Neither AG nor UAG had any effect in healthy subjects. However, systemic administration of UAG, but not AG, prevented diabetes-induced EPC damage by modulating the NADPH oxidase regulatory protein Rac1 and improved the vasculogenic potential both in individuals with type 2 diabetes and in *ob/ob* mice. In addition, unlike AG, UAG facilitated the recovery of bone marrow EPC mobilization. Crucial to EPC mobilization by UAG was the rescue of endothelial NO synthase (eNOS) phosphorylation by Akt, as UAG treatment was ineffective in eNOS knockout mice. Consistently, EPCs expressed specific UAG-binding sites, not recognized by AG.

CONCLUSIONS—These data provide the rationale for clinical applications of UAG in pathologic settings where AG fails. ***Diabetes*** **59:1016–1025, 2010**

Ghrelin is a 28–amino acid peptide that circulates in both acylated (AG) and, more abundantly, unacylated forms (UAG) (1). Historically, AG, usually referred to as ghrelin, has been considered the only active form of the peptide. It recognizes the Gq-coupled growth hormone secretagogue receptor type 1a, denoted as GHS-R1a (2), mediating its growth hormone–releasing properties as well as other significant neuroendocrine actions (3). In contrast, UAG does not bind the GHS-R1a and is devoid of growth hormone secretagogue activity (3). Nevertheless, both AG and UAG share affinity for common binding sites that mediate vascular activities in terms of vasodilation and inhibition of cardiomyocyte and endothelial cell apoptosis (3). Furthermore, UAG effects, different from those elicited by AG, have also been demonstrated (4), suggesting the existence of an additional unidentified receptor for UAG.

Besides expression in several tissues (5), including the cardiovascular system (6,7), ghrelin is produced mostly by the stomach. In particular, in humans, AG reduces insulin sensitivity and exerts orexigenic activity, whereas UAG has opposite effects (8). Circulating total ghrelin levels are negatively associated with BMI (9), and ghrelin secretion is reduced in obese (10) and type 2 diabetic (11) individuals, possibly as a compensatory mechanism protecting against hyperglycemia. Interestingly, a relative excess of AG compared with UAG has been reported in clinical conditions marked by insulin resistance (12), raising the possibility that the altered UAG/AG ratio could play a role in the altered glucose metabolism and its ongoing complications.

Among such complications, accelerated vascular disease is widely recognized as the major cause of disability and death in individuals with type 2 diabetes. Endothelial injury is thought to represent a crucial step in the initiation and progression of atherosclerotic vascular disease in this setting (13). Previous data support the central role of advanced glycosylation end products (AGEs) (14) and of NADPH oxidase–mediated reactive oxygen species (ROS) production in impaired vascular remodeling associated with diabetes (15). NADPH oxidase (Nox) consists of a membrane-bound catalytic subunit and several cytosolic regulatory subunits ($p47^{phox}$ and $p67^{phox}$). Moreover, the GTPase-bound Rac1 is required for the functional assembly of the holoenzyme (15).

Vascular remodeling relies on resident endothelial cells and on circulating endothelial progenitor cells (EPCs): early circulating angiogenic cells (CACs) and late EPCs (16,17). Although they share several common features, they have distinct features with respect to morphology, proliferative potential, and functional characteristics (18). Compelling evidence indicates that changes in their number and functional activities are closely associated with cardiovascular risk factor profiles (19,20), impacting on their delivery to sites of ischemia where angiogenesis might be required. Indeed, treatment with certain cytokines to induce bone marrow mobilization of EPCs has been shown to be cardioprotective (21). EPC mobilization strictly depends on local secretion and activation of the matrix metalloproteinase 9 (MMP9) in the hematopoietic and stromal compartments of the bone marrow (22). In

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Received 8 June 2009 and accepted 3 January 2010. Published ahead of print at http://diabetes.diabetesjournals.org on 12 January 2010. DOI: 10.2337/db09-0858.

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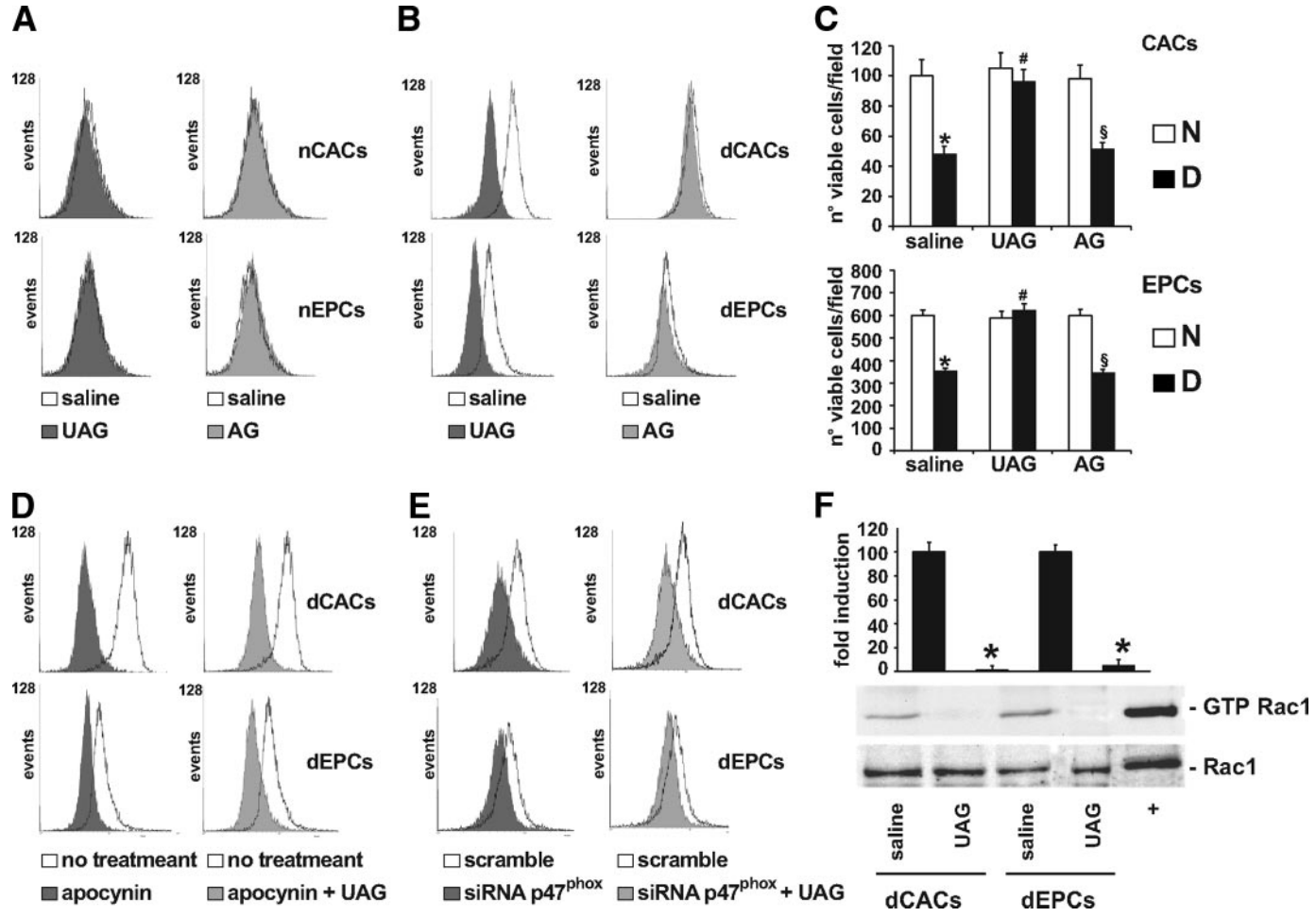

FIG. 1. UAG, by preventing Rac1 activation, protects CACs and EPCs from ROS production. *A* and *B*: Dichlorofluorescein diacetate assay was performed on CACs or EPCs, recovered from healthy subjects (nCACs, nEPCs) or from diabetic patients (dCACs, dEPCs) treated with saline, UAG, or AG (*white area:* no treatment; *dark gray area:* UAG infused; *light gray area:* AG infused). *C*: CACs and EPCs isolated from healthy donors (N) and from diabetic patients (D) treated as above were cultured as described in the RESEARCH DESIGN AND METHODS section. Trypan blue–excluded viable cells were counted (* and § indicate $P < 0.05$, healthy donors vs. diabetic groups; #$P < 0.05$, saline vs. UAG infusion). *D*: ROS production, in response to apocynin alone or in combination with UAG, was evaluated on dCACs and dEPCs isolated from saline-treated patients (*white area:* no treatment; *dark gray area:* apocynin; *light gray area:* apocynin + UAG). *E*: ROS production was evaluated on dCACs and dEPCs isolated from saline-infused patients and transfected with the scrambled sequence or with p47^{phox} siRNA. Cells were treated or not with UAG (*white area:* scramble; *dark gray area:* p47^{phox} siRNA; *light gray area:* p47^{phox} siRNA + UAG). *F*: dCACs and dEPCs retrieved from saline- or UAG-infused patients were analyzed for Rac1 activation. Cell extracts were either pulled down with GST-PAK or directly subjected to SDS-PAGE. Eluates or extracts were immunoblotted with an anti-Rac1 antibody. GTP-Rac1 and total Rac1 are indicated. Interleukin-3–treated endothelial cells were used as positive control (+). CACs and EPCs were recovered after a 6-h infusion with saline, UAG, or AG as indicated.

turn, activated MMP9 converts the membrane-bound form of the Kit ligand (mbKitL) into a soluble form (sKitL) that promotes hematopoietic and endothelial progenitor cell proliferation and facilitates their mobilization into the circulation (22). Because an impairment of progenitor cell mobilization has also been reported in mice lacking endothelial NO synthase (eNOS) (23), eNOS may also be involved in the control of progenitor cell delivery to sites of neovascularization. Consistently, pathologic settings, characterized by reduced systemic NO bioavailability, also show defective EPC mobilization and compromised vascular regenerative processes (24,25).

Based on previous evidence for cardiovascular protective effects of both AG and UAG, the aim of the present study was to investigate the therapeutic potential of ghrelin isoforms in diabetes-associated vascular disease and related mechanisms. Herein, we demonstrate that UAG, but not AG, systemic administration protects diabetic EPCs from senescence and restores their vasculogenic potential by regulating the small GTPase Rac1 activity. Finally, we show that UAG, unlike AG, rescues defective EPC mobilization in individuals with type 2 diabetes, but has no effect in healthy subjects.

RESEARCH DESIGN AND METHODS

Patients and control subjects. Blood was recovered from 14 individuals with type 2 diabetes who arrived in our patient clinic (sex [M/F], 8/6; A1C, 8 ± 1.2%; age, 55.8 ± 9.46 years; BMI, 28.1 ± 3.22 kg/m^2; creatinine, 1.06 ± 0.20 mg/dl; waist circumference, 98.8 ± 8.02 cm; total cholesterol, 198 ± 30 mmol/l; HDL cholesterol, 48 ± 12.98 mmol/l; LDL cholesterol, 115 ± 38.94 mmol/l; triglycerides, 159.5 ± 55.23 mg/dl; fasting glucose, 125 ± 18.03 mg/dl; no retinopathy; hypertension in three patients; blood pressure, 142/89 mmHg; cholesterol/apolipoprotein B, 1.3 ± 0.3). All were treated only with diet. No medications were used. A total of 12 blood donors were used as control subjects (sex [M/F], 6/6; age, 47.8 ± 4.85 years; BMI, 21.25 ± 8.07 kg/m^2; creatinine, 0.90 ± 0.083 mg/dl; total cholesterol, 164.03 ± 9.68 mmol/l; HDL cholesterol, 50 ± 8.4 mmol/l; LDL cholesterol, 85.26 ± 16.72 mmol/l; triglycerides, 131.14 ± 25.03 mg/dl; no retinopathy; no hypertension; blood pressure, 126/70 mmHg; cholesterol/apolipoprotein B, 1.6 ± 0.2). Ethics approval was obtained from both SIMT (Servizio Immunoematologia e Medicina Trasfusionale) and the Institutional Review Board of S. Giovanni Battista Hospital, Turin, Italy. Informed consent was provided according to the Helsinki

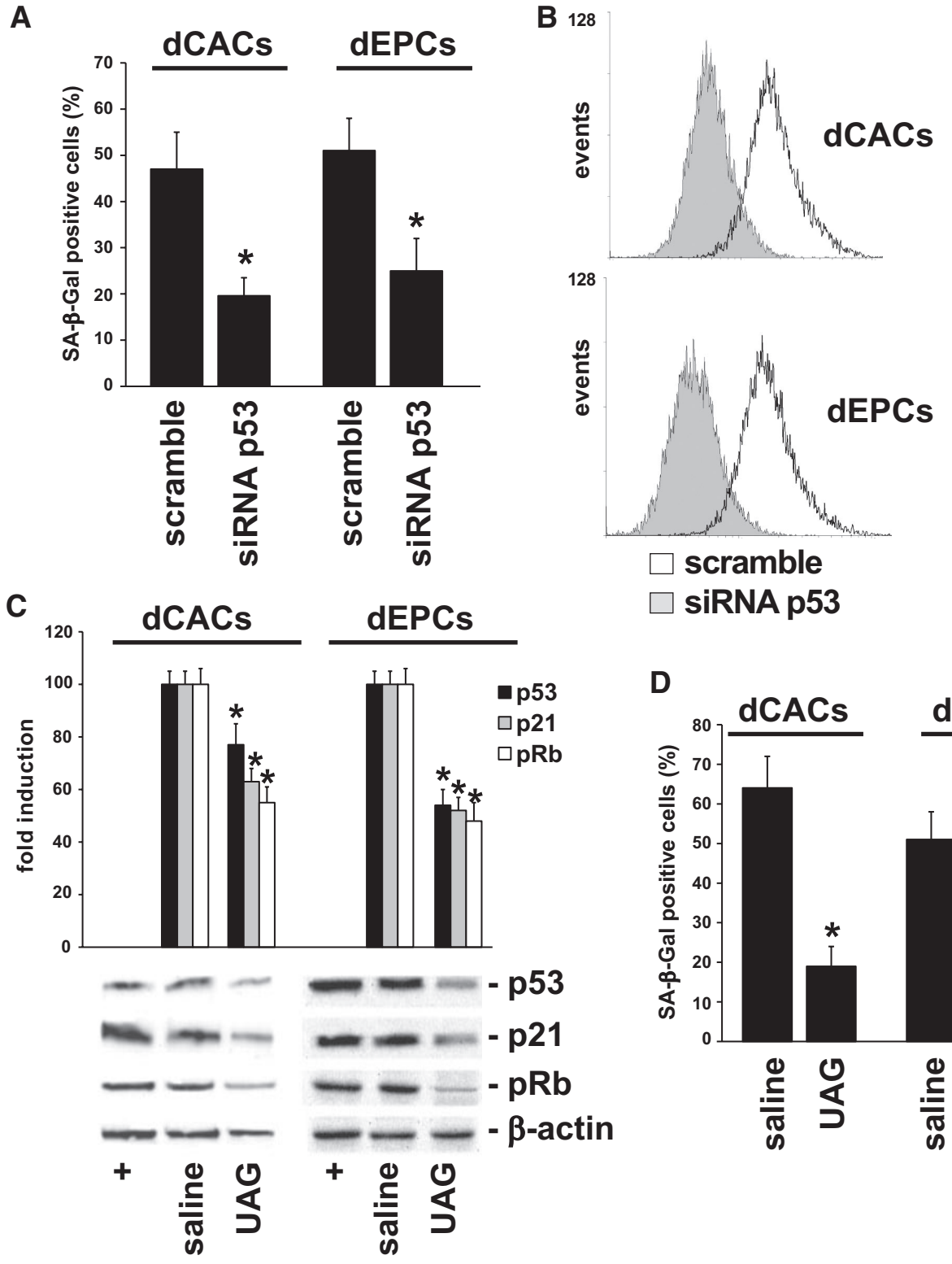

FIG. 2. UAG prevents dCAC and dEPC senescence. *A*: Senescence was evaluated on dCACs and dEPCs recovered from saline-infused patients transfected with pSUPER retro containing p53 siRNA or the scrambled sequence as control and expressed as the percentage of SA-β-gal–positive cells (**P* < 0.05, scramble vs. p53 siRNA). *B*: ROS production was evaluated on dCACs and dEPCs recovered from saline-infused patients transfected with pSUPER retro containing p53 siRNA or the scrambled sequence (*white area:* scramble; *gray area:* p53 siRNA). *C*: dCACs and dEPCs retrieved from saline- or UAG-infused patients were lysed and analyzed for p53, p21, and pRb expression by Western blotting. Oxidized LDL–treated endothelial cells were used as positive control (+). *D*: Senescence was evaluated on dCACs and dEPCs recovered from saline- or UAG-infused patients and expressed as the percentage of SA-β-gal–positive cells (**P* < 0.05, saline vs. UAG infusion).

Declaration. For the present study, we had no direct contact with human subjects.

Testing sessions were as follows: UAG (3.0 μg · kg^{-1} · h^{-1} i.v. as infusion for 12 h, from 0 to 12 h); AG (1.0 μg · kg^{-1} · h^{-1} i.v. as infusion for 12 h, from 0 to 12 h); and isotonic saline (infusion from 0 to 12 h).

All tests were performed starting at 8:30–9:00 A.M. after overnight fasting. An indwelling catheter was placed into an forearm vein for slow infusion of isotonic saline. Cells were isolated from blood samples taken at 0, 6, and 12 h.

Isolation, characterization, and culture of early EPC (CACs) and late EPCs (EPCs) from peripheral blood mononuclear cells. To isolate CACs, peripheral blood mononuclear cells retrieved from healthy subjects (nCACs) or from individuals with type 2 diabetes (dCACs) were plated on fibronectin-coated dishes as described by Hill et al. (19). Briefly, the cells were cultured for 4 days in EGM-2 medium (Cambrex, Walkersville, MD). To isolate EPCs (nEPCs from healthy subjects, dEPCs from individuals with type 2 diabetes), peripheral blood mononuclear cells were recovered and cultured onto collagen-1–coated dishes for 21 days in EGM-2 medium as described by Yoder et al. (26). In selected experiments, CACs or EPCs recovered from saline-infused healthy subjects were cultured with 1.2 mg/ml AGE, H_2O_2 (100 μmol/l), 5 mmol/l glucose, or 25 mmol/l glucose alone or in combination with 1 μmol/l UAG or 1 μmol/l AG; 1 μmol/l UAG or 1 μmol/l AG was also used alone. Fluorescence-activated cell sorter analysis was used to characterize CAC and EPC surface markers (anti-CD45, anti-CD14, anti-CD34, anti-CD31, anti–Tie-2, anti-KDR, anti-vWF antibodies; see supplementary methods, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0858/DC1). eNOS expression was also evaluated.

Diabetic and control mice. The plasma glucose and insulin determination for each group of mice (Charles River Lab, Lecco, Italy) are reported: 16 8-week-old *ob/ob* mice (blood glucose, 296 ± 19.6 mg/dl; insulin, 55 ± 9 ng/ml); 16 8-week-old C57BL/6J wild-type mice (blood glucose, 92 ± 7.2 mg/dl; insulin, 10 ± 0.5 ng/ml). Animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (27). Blood glucose was measured with a One Touch II glucose meter (LifeScan, Mountain View, CA). Serum insulin was measured with a mouse insulin radioimmunoassay kit (Linco Research, St. Charles, MO), following the manufacturer's instructions.

Reagents and antibodies. All reagents and antibodies used are reported in the supplementary methods.

Detection of ROS; GTP-Rac1 loading assay; senescence assay; Western blot analysis; silencing of endogenous p53, Akt, and p47^{phox} by small interfering RNAs (siRNAs); Matrigel plug assay; immunohistochemistry and immunofluorescence; human and mouse mobilization assays; enzyme-linked immunosorbent assay and radioimmunoassay; isolation and culture of bone marrow–derived cells; and evaluation of MMP9 activation, cytofluorimetry analysis, and in vitro migration assays were described in detail in the supplementary methods.

Statistical analysis. In vitro and in vivo results are representative of at least three independent experiments, performed at least in triplicate. Densitometric analysis using a Bio-Rad GS 250 molecular imager was used to calculate the differences in the fold induction of protein activation or expression. Significance of differences between experimental and control values (*, #, °, and § indicate $P < 0.05$, statistically significant) was calculated using ANOVA with Newman-Keuls multicomparison test.

RESULTS

UAG protects diabetic EPCs from oxidative stress by regulating Rac1. Several lines of evidence indicate that the number and function of EPCs are impaired in diabetes (17,19) and that these events rely mainly on Nox-mediated ROS production (15). The effect of in vivo UAG and AG administration in protecting both CACs and EPCs from oxidative damage was first evaluated. Toward this end, cells isolated from UAG- or AG-treated individuals with type 2 diabetes and healthy subjects were characterized for CAC and EPC markers (supplementary Fig. 1) (18) and subjected to dichlorofluorescein diacetate fluorescence assay. The results reported in Fig. 1*A* and *B* demonstrate that UAG, but not AG, treatment (6 h) drastically reduced ROS production in individuals with type 2 diabetes. Moreover, the number of viable cells was significantly higher after UAG treatment compared with before (Fig. 1*C*). A protective effect of UAG on ROS production was also demonstrated in cells cultured with AGE or high glucose (supplementary Fig. 2*A*). Similar results were obtained

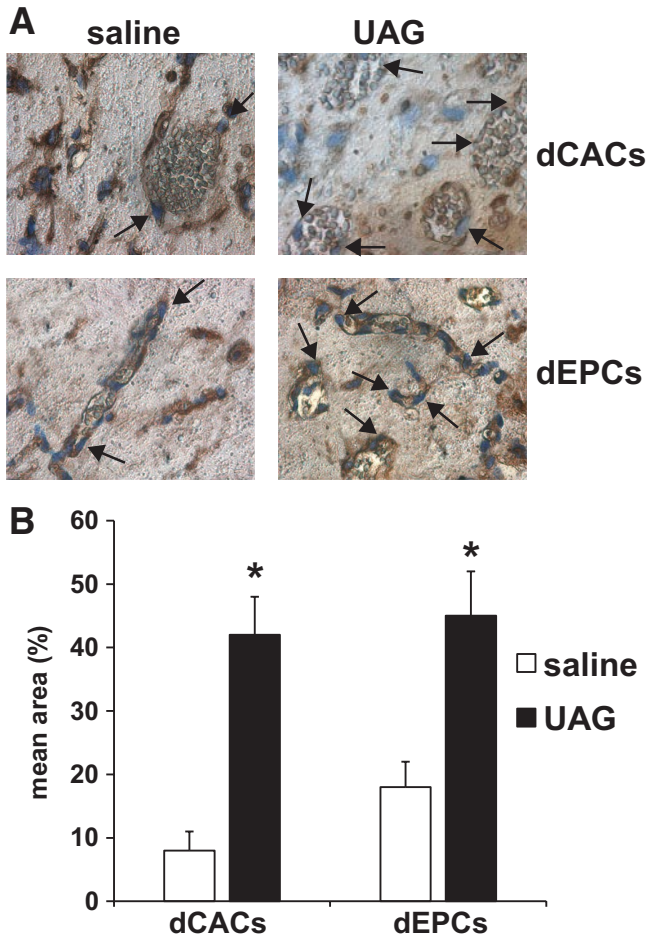

FIG. 3. UAG increases de novo vessel formation. *A*: Immunohistochemistry of representative functional vessels formed by labeled dCACs and dEPCs, recovered from saline-infused (*left*) or from UAG-infused (*right*) diabetic patients. Black arrows indicate labeled cells (×40 magnification). *B*: Quantification of newly formed vessels was expressed as percentage ± SD of the vessel area to the total Matrigel area (*$P < 0.05$, saline infused vs. UAG infused). (A high-quality digital representation of this figure is available in the online issue.)

when H_2O_2 was used (supplementary Fig. 2*B*). These findings, together with the observation that neither in vitro nor in vivo UAG administration (supplementary Fig. 2*C* and *D*) changed AGE receptor (RAGE) expression, suggest that effector(s) downstream of RAGE is the target for protective effect of UAG. Because similar results were obtained after 12 h of treatment (data not shown), data throughout the study relates to 6-h UAG treatment.

Apocynin, known to affect the assembly of Nox subunits (28), was used to investigate the role of Nox in regulating ROS production. Cells recovered from saline-infused individuals with type 2 diabetes were subjected to apocynin treatment. ROS production was prevented in both dCACs and dEPCs (Fig. 1*D*). UAG addition could not further enhance apocynin's effects (Fig. 1*D*), suggesting that Nox activity might be controlled by UAG. As the assembly of p47^{phox} and p67^{phox} subunits is required for Nox enzymatic activity (29), silencing of p47^{phox} in dCACs and dEPCs (supplementary Fig. 3*A*) prevented ROS production (Fig. 1*E*). Once more, this effect could not be further enhanced by the addition of UAG (Fig. 1*E*). Nox2 activity is also dependent on the small GTPase Rac (29). ROS generation in response to AGE (supplementary Fig. 3*B* and *C*) was prevented in cells expressing a dominant-negative RacN17

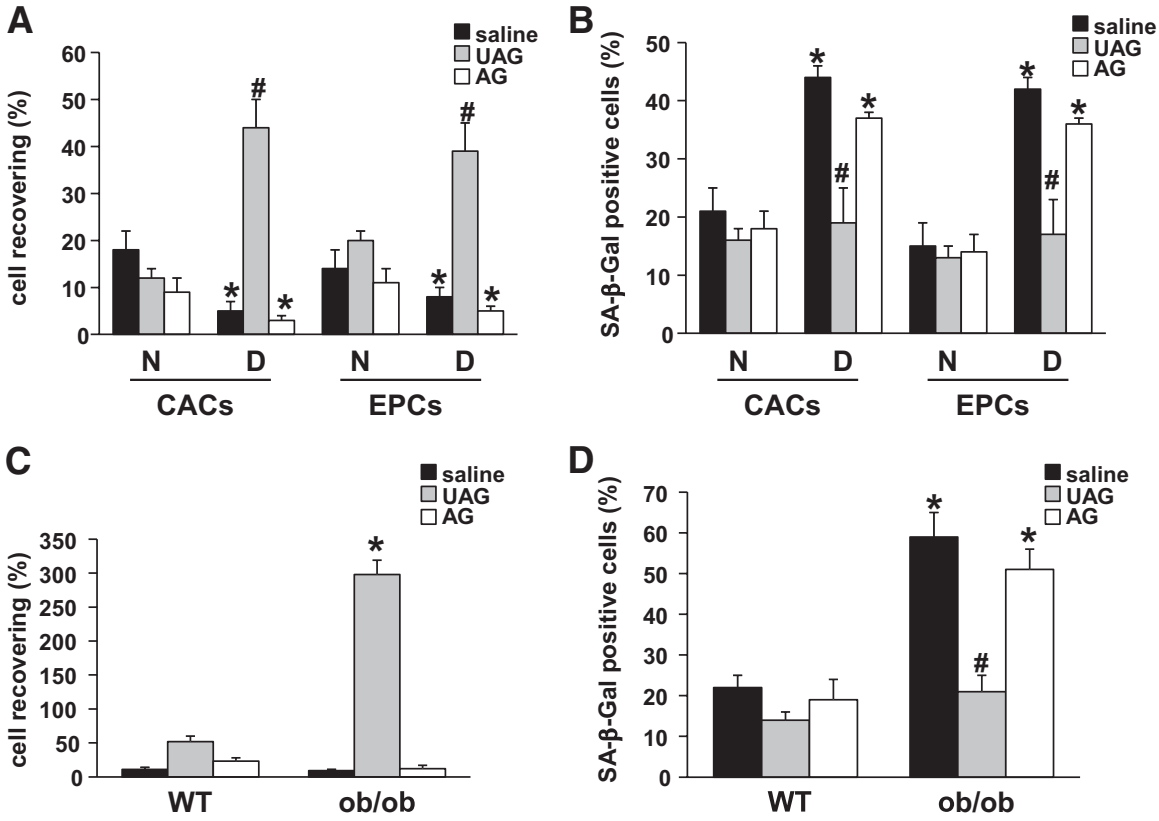

FIG. 4. UAG induces CAC and EPC mobilization. ***A*****: Percentage of CACs and EPCs recovered after 6 h of saline, UAG, or AG treatment: (N) healthy donors and (D) diabetic patients (*$P < 0.05$, diabetic patients vs. healthy donors, #$P < 0.05$ saline vs. UAG infusion).** ***B*****: Senescence was evaluated on CACs and EPCs recovered as described in** ***A*** **and expressed as the percentage of SA-β-gal–positive cells (*$P < 0.05$, diabetic patients vs. healthy donors, #$P < 0.05$ saline vs. UAG infusion).** ***C*****: Percentage of EPCs recovered from wild-type or** ***ob/ob*** **mice infused for 12 h with saline, UAG, or AG (*$P < 0.05$ WT UAG-infused vs.** ***ob/ob*** **UAG-infused mice).** ***D*****: Senescence was evaluated on murine EPCs treated as described in** ***C*** **and expressed as the percentage of SA-β-gal–positive cells (*$P < 0.05$, WT vs.** ***ob/ob*** **mice, #$P < 0.05$ saline vs. UAG infusion). All data are the mean ± SD obtained by three individual investigators.**

construct. We thus hypothesized that UAG interfered with Rac1 activity. Indeed, Rac1 activation was detected in cells recovered from saline-treated individuals with type 2 diabetes, but not from UAG-infused patients (Fig. 1*F*). Similar results were obtained in vitro by culturing cells with AGE (supplementary Fig. 3*D*). Hence, the modulation of Rac1 activity is a crucial step in the UAG anti-ROS protective effect.

Rac1 membrane localization and function rely on isoprenylation, which has been correlated with AMP-activated protein kinase (AMPK)-dependent hydroxymethylglutaryl CoA reductase activity (30). To rule out the possibility that inhibition of Rac1 activity by UAG depends on this pathway, AMPK phosphorylation was evaluated. Neither short- nor long-term exposure to UAG alone or in combination with AGE affected AMPK activity (supplementary Fig. 4).

UAG prevents cell senescence and improves de novo vessel formation. p53, p21, and pRb are major regulators of cell senescence (31). The above results prompted us to evaluate whether the increase in ROS production, generally considered as an upstream signal, translates into an accelerated onset of senescence and whether UAG could rescue this effect. Because both senescence and ROS generation (Fig. 2*A* and *B*) were prevented by silencing p53 (supplementary Fig. 5*A*), we investigated the in vivo effect of UAG on p53 expression. Accordingly, UAG treatment was able to prevent p53 accumulation, p21 expression, and Rb phosphorylation (Fig. 2*C*) and to reduce the number of senescence-associated β-galactosidase (SA-β-gal)-positive dCACs and dEPCs (Fig. 2*D*) in UAG-challenged patients. Similar results were obtained in AGE-treated cells (supplementary Fig. 5*B* and *C*).

To assess whether the protective effect of UAG also resulted in an enhancement of dCAC and dEPC vasculogenic capability, de novo vessel formation was analyzed in severe combined immunodeficient mice injected with cells recovered from UAG-treated individuals with type 2 diabetes. At 15 days after injection, plugs were recovered and analyzed by immunohistochemistry. As shown in Fig. 3*A* and *B*, the number of functional vessels formed by cells recovered from UAG-treated patients was significantly increased with respect to those from saline-treated patients. The origin of neovessels from host vasculogenic cells was excluded because the majority of vessels were lined by human HLA class I–positive cells (supplementary Fig. 6) (32). Thus, these data provide evidence that UAG restores dCAC and dEPC vasculogenic activity.

UAG improves EPC mobilization. Defective EPC and CAC mobilization has been reported in diabetes (19,20). To further investigate the potential therapeutic effect of UAG, CACs and EPCs were recovered from 10 normal healthy subjects and 10 individuals with type 2 diabetes, characterized, and counted. UAG treatment led to an increase in the number of recovered cells in individuals with type 2 diabetes compared with that of healthy subjects, and no effect of UAG treatment was detected in healthy subjects (Fig. 4*A*). In contrast, no differences

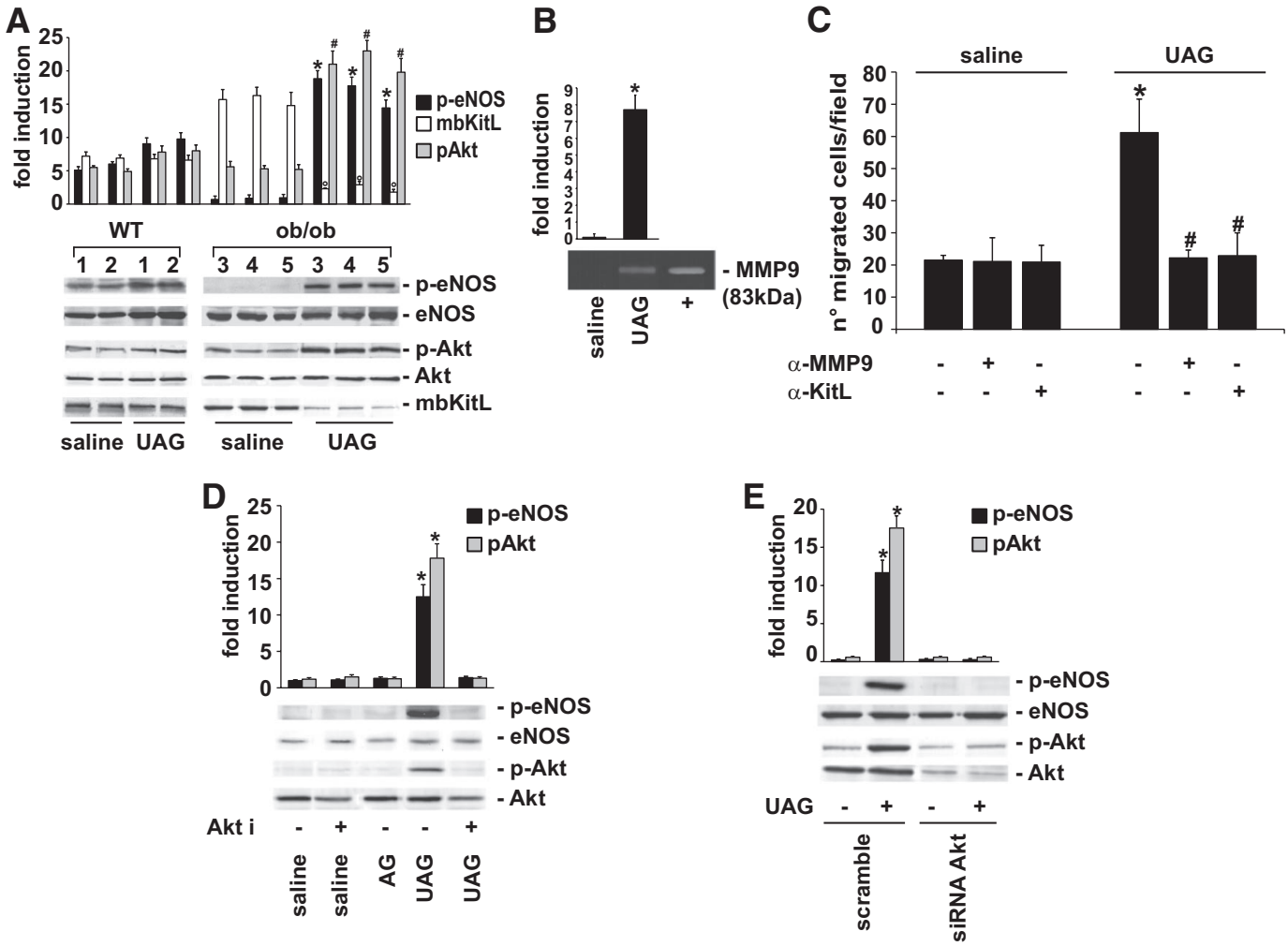

FIG. 5. UAG restores eNOS phosphorylation. *A*: Adherent cells (from total bone marrow populations) obtained from saline-, UAG-, or AG-treated WT or *ob/ob* mice were lysed and analyzed by Western blotting using the indicated antibodies. The blots are representative of two WT (1–2) or three *ob/ob* (3–5) samples. Interleukin-3–treated endothelial cells were used as positive controls. *B*: Representative zymogram of MMP9 gelatinolytic activity on serum recovered from saline- or UAG-treated mice. As positive control, human serum was used (+). *C*: Migration assays performed on bone marrow cells using sera recovered from saline- or UAG-treated *ob/ob* mice. MMP9 or sKitL neutralizing antibodies were added where indicated (*$P < 0.05$, saline vs. UAG, #$P < 0.05$, UAG vs. UAG + anti-MMP9 and UAG + anti-sKitL). *D*: Adherent cells obtained from *ob/ob* total bone marrow pools were treated as indicated for 40 min and processed for Western blotting using the indicated antibodies. *E*: Adherent cells from *ob/ob* total bone marrow pools were depleted (siRNA Akt) or not (scrambled) of Akt, subjected to UAG treatment, and analyzed by Western blotting using the indicated antibodies.

between AG- or saline-treated healthy and type 2 diabetic individuals were observed (Fig. 4*A*). In addition, as shown in Fig. 4*B*, the percentage of circulating senescent dCACs and dEPCs decreased upon UAG treatment.

Circulating stromal derived factor-1 (SDF-1) (33) and vascular endothelial growth factor (VEGF) (34) strictly control progenitor cell mobilization under stress conditions. We herein demonstrate that 6- or 12-h (data not shown) UAG or AG infusion did not change their serum concentrations (supplementary Fig. 7). Similarly, IGF-1 (35) serum concentration was not affected by UAG treatment (supplementary Fig. 7).

UAG mobilizes EPCs by rescuing eNOS activity. For validation and characterization of the molecular mechanisms regulating bone marrow mobilization, a mouse model of type 2 diabetes (*ob/ob* mice) was used. After treatment with saline, UAG, or AG, recovered cells were subjected to fluorescence-activated cell sorter analysis for surface markers to confirm EPC identity (data not shown). UAG treatment induced a strong increase of recovered EPCs only in *ob/ob* mice (Fig. 4*C*). Finally, the number of senescent cells was significantly lower in UAG-treated *ob/ob* mice compared with untreated or AG-treated animals (Fig. 4*D*), reproducing our findings in human subjects.

As an impairment of eNOS phosphorylation contributes to defective EPC mobilization in the diabetic setting (36), we investigated whether UAG modulated eNOS activity and the activation of its regulatory protein, Akt (37), in bone marrow stromal cells. The stromal origin of the eNOS-expressing cells was confirmed by the presence of mbKitL (Fig. 5*A*). In *ob/ob* mice, UAG treatment restored both eNOS and Akt phosphorylation (Fig. 5*A*). Consistent with the pivotal role of a local activation of MMP9 in promoting progenitor cell mobilization (22), gelatin zymography revealed that MMP9 gelatinolytic activity was induced by UAG (Fig. 5*B*). The role of MMP9 activation and sKitL release in controlling this event was further confirmed by functional studies using anti-MMP9 and anti-KitL neutralizing antibodies (Fig. 5*C*). Accordingly, in parallel with effects on MMP9 activation, expression of the mbKitL was decreased in *ob/ob* mice subjected to UAG

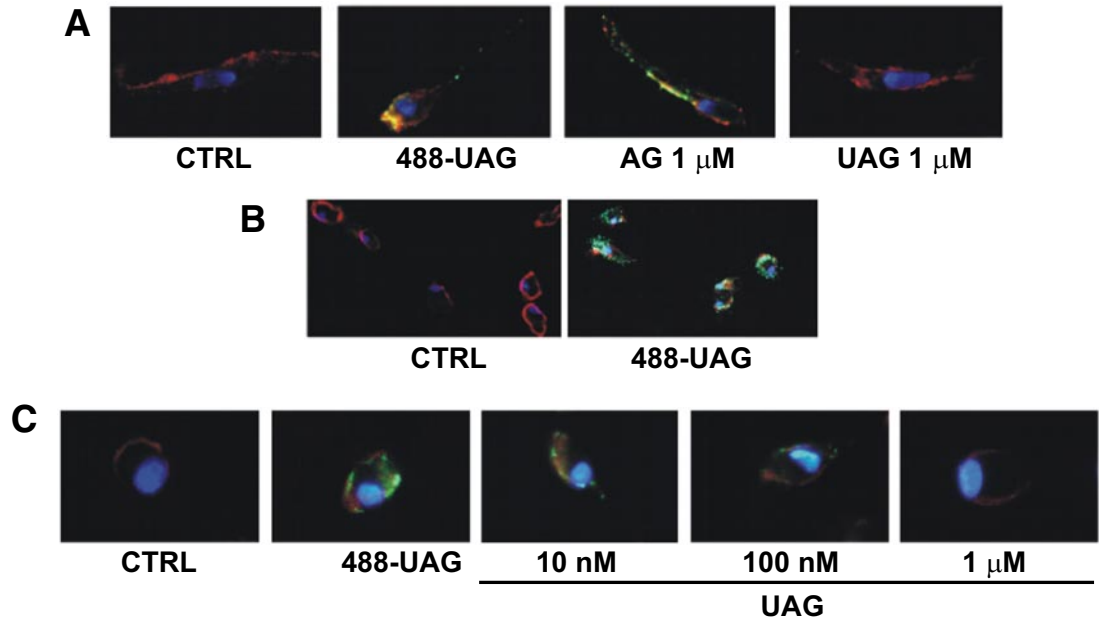

FIG. 6. Visualization of UAG-binding sites on CACs by 488-UAG. *A–C:* Living cells were incubated in the absence (CTRL) or presence of 100 nmol/l 488-UAG with or without the indicated ligands. The putative receptor is stained in green (488-UAG); the plasma membrane in red (phycoerythrin-CD45); and the nucleus in blue (DAPI). *A*: Visualization of 488-UAG–binding site distribution on CAC plasma membrane. CAC incubation with ligands was carried out at 4°C for 4 h. 488-UAG was displaced by 1 μmol/l UAG but not by 1 μmol/l AG (×40 magnification). *B* and *C*: Visualization of 488-UAG–binding distribution on CACs upon incubation with the indicated ligands for 20 min at 25°C. *B*: Representative photomicrograph showing the 488-UAG–labeled cell surface binding sites internalized into endocytotic vesicles (×20 magnification). *C*: 488-UAG binding and internalization were completed by increasing concentrations of the unlabeled UAG. (A high-quality digital representation of this figure is available in the online issue.)

treatment (Fig. 5*A*). Although we cannot rule out the possibility that a paracrine effect of UAG occurs in vivo, herein we have shown that in vitro UAG treatment for 40 min elicited Akt and eNOS phosphorylation in stromal cells obtained from *ob/ob*-derived total bone marrow pools (Fig. 5*D*). In agreement with the results measuring EPC mobilization, AG failed to induce Akt and eNOS phosphorylation (Fig. 5*D*). The finding that UAG failed to induce eNOS phosphorylation after knocking down Akt (Fig. 5*E*) or inhibiting its activation (Akt inhibitor) (Fig. 5*D*) indicates that Akt is a key modulator of the UAG effect. The above data were validated by the lack of effect of UAG treatment in NOS3$^{-/-}$ mice (23) (supplementary Fig. 8).

CACs express specific UAG-binding sites. Based on our collective dataset, we further investigated whether the biological response of CACs to UAG was mediated by specific binding sites localized to the plasma membrane. To this end, double immunofluorescence experiments, using the UAG analog 488-UAG (100 nmol/l) to label putative binding sites and anti–phycoerythrin-CD45 antibody as a membrane marker, were carried out at 4°C. As shown in Fig. 6*A*, at 4°C 488-UAG–binding sites colocalized with CD45, indicating a plasma membrane localization. In agreement with the functional data, unlabeled UAG (1 μmol/l) displaced the fluorescent signal from the cell surface, whereas AG (1 μmol/l) did not (Fig. 6*A*). In addition, we also monitored receptor activation at 25°C. As shown in Fig. 6*B*, after 20-min stimulation, receptor clusters undergoing internalization were visualized as labeled cytoplasmic vesicles. Moreover, increasing concentrations of unlabeled UAG specifically displaced the fluorescent ligand from both the plasma membrane and endocytic vesicles (Fig. 6*C* and supplementary Fig. 9). Similar results were obtained using EPCs (data not shown).

DISCUSSION

The present data first demonstrate that UAG, unlike AG, reverts diabetes-induced EPC damage by inhibiting activation of the Nox regulatory protein Rac1; as a consequence, UAG protects diabetic EPCs from senescence and improves their vasculogenic capability; again, only UAG rescues EPC mobilization under diabetic conditions by restoring eNOS phosphorylation, and specific UAG-binding sites mediate its effects.

UAG is the most abundant circulating form of ghrelin (3) and plays a positive role on glucose metabolism. In contrast, basic and clinical studies have proposed AG as a diabetogenic hormone (8,38). Indeed, clinical conditions of insulin resistance are associated with an alteration in the circulating ghrelin profile with relative AG excess with respect to UAG (12). Thus, it is tempting to speculate that an altered UAG/AG ratio might contribute both to metabolic changes and to diabetes-associated complications.

Among diabetes-associated complications, abnormal vascular remodeling is believed to play a major role in accelerating vascular disease (39). Historically, it has been assumed that new blood vessels originate from sprouting cells and co-opting of neighboring preexisting vessels. However, both physiological and pathologic angiogenesis are also supported by mobilization and recruitment of other cell types, including the bone marrow–derived cells, such as EPCs (16,18,40,41). Interestingly, alterations in the number and function of these cells correlate with the risk factor profile (19,20). Overproduction of ROSs in these pathologic settings seems to contribute to impaired vascular regenerative processes (42). The plasma membrane Nox is recognized as one of the major regulators of ROS generation (15,29). Activation of the enzyme can occur via many upstream signaling pathways converging on phos-

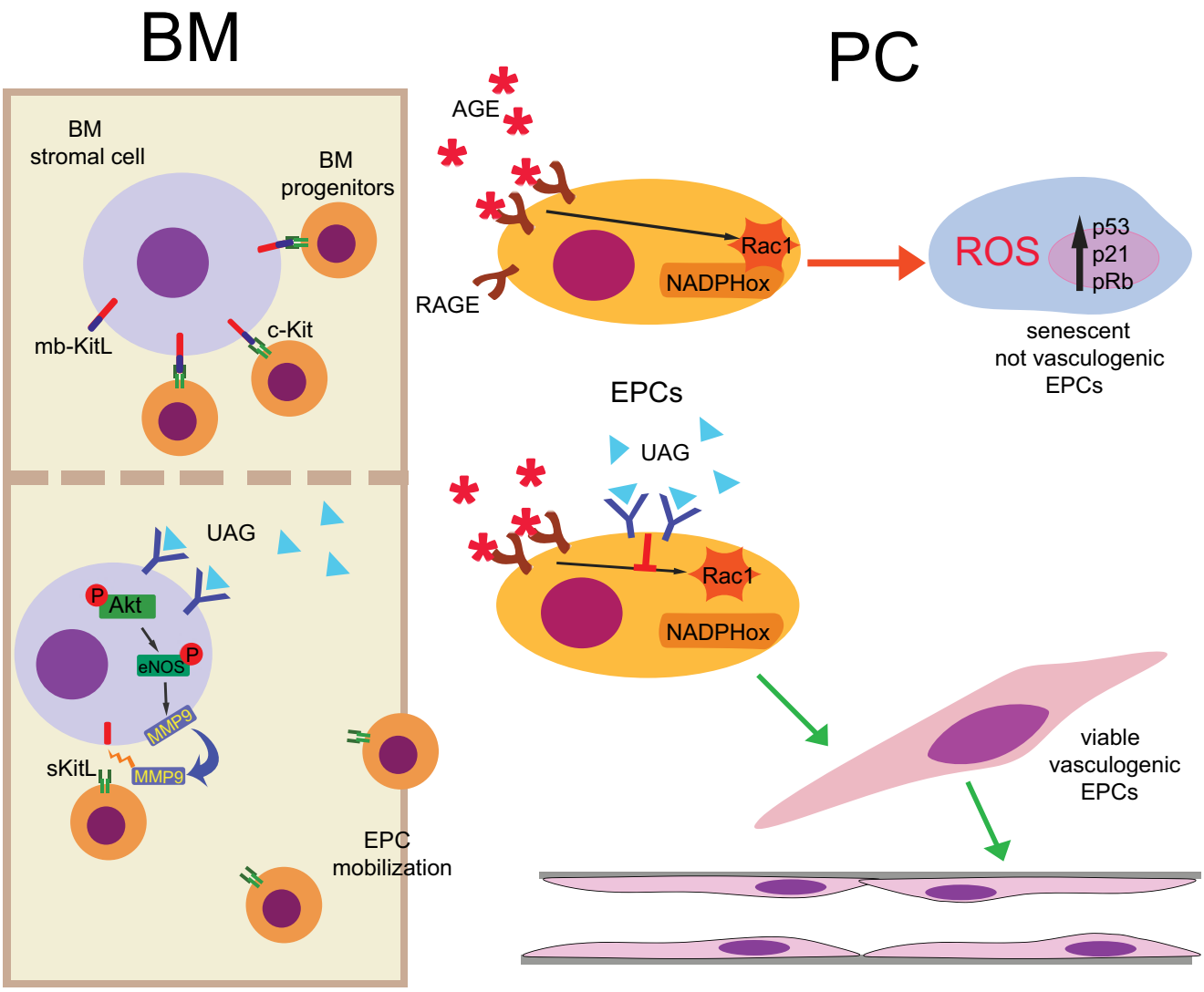

FIG. 7. Schematic model for UAG impact on EPC physiology. The resident bone marrow pool of early and late EPCs was mobilized into circulation after UAG-mediated Akt activation in stromal cells, which leads to eNOS phosphorylation and MMP9 activation. In turn, MMP9 activation switched on the release of sKitL, a determinant for EPC exit into the bloodstream. In addition, UAG treatment restores diabetic viability of EPCs and increases their vasculogenic capability by preventing ROS generation and its downstream signaling pathway (p53 accumulation, p21 expression, Rb phosphorylation). This effect results from UAG-mediated inhibition of Rac1 activation, a prerequisite for NADPH oxidase assembly. BM, bone marrow compartment; PC, peripheral compartment.

phorylation of $p47^{phox}$ and activation of Rac1 leading to the oxidase assembly (29). Our study shows that UAG can protect diabetic EPCs from oxidative stress by affecting the Nox regulatory protein Rac1.

The accelerated onset of senescence contributes to the impaired EPC bioavailability in patients with diabetes (17). The tumor suppressor p53 is a transcription factor involved in DNA damage mechanisms and is recognized as a negative regulator of cell proliferation in human atherosclerotic and restenosis lesions (43). Moreover, the p53-mediated pathway contributes to EPC senescence-like growth arrest in diabetes (44). Accordingly, the present study shows that silencing p53 in EPCs isolated from individuals with type 2 diabetes or cultured with AGE prevents both ROS production and senescence. The finding that both in vitro and in vivo exposure to UAG negatively modulates p53 accumulation identifies the p53-mediated signal as the primary mechanism through which UAG protects EPCs from senescence. We also found that, by preventing oxidative stress, UAG improves de novo vessel formation. The efficacy of cell therapy certainly depends on the number, functional capability, and successful retention of cells in the site of action. Thus, our data strongly suggest that naturally occurring UAG induces improvement of EPC function and survival that may translate into a more efficient response to vascular dysfunction.

Senescence is also associated with impaired mobilization of bone marrow–derived cells (17). The molecular interactions between stem cells and bone marrow stromal cells, and the molecular mechanisms controlling their mobilization in the bone marrow microenvironment, are poorly understood (45). Considerable interest has arisen about agents able to mobilize and augment progenitor cell delivery to sites of vascular injury to enhance revascularization. Among these, SDF-1 and VEGF are recognized as primary regulators of bone marrow cell mobilization during stress conditions (22,46). In addition, under physiological stresses, the activation of matrix proteases within the bone marrow microenvironment results in the release of

sKitL, which enables endothelial and hematopoietic progenitor cells to transit from the quiescent to the proliferative niche and facilitates their mobilization into the circulation (22). The delivery of progenitor cells to sites of neovascularization also relies on functional eNOS activity (23). Indeed, in pathologic settings associated with blunted eNOS activity and reduced systemic NO bioavailability, defective EPC mobilization and impaired vascular regenerative processes occur (24,25). eNOS activation through Akt has been reported for AG acting on the Gq-coupled GHS-R1a in cultured endothelial cells (47). We herein demonstrate that UAG can restore eNOS activity via Akt-mediated phosphorylation in a pathologic setting characterized by impaired eNOS phosphorylation. This was particularly true in bone marrow stromal cells from diabetic mice. Such an event was found crucial to EPC bone marrow mobilization, because UAG had no effect in eNOS knockout mice. Gu et al. (48) identified MMP9 as a major target of NO. We also showed that eNOS phosphorylation, occurring in response to UAG, is associated with MMP9 activation and possibly with the release of sKitL, as suggested by the reduced expression of the mbKitL on stromal cells recovered from UAG-treated mice and by functional studies. Although we cannot exclude that UAG may act in a paracrine manner by locally inducing the release of VEGF from bone marrow stromal cells, we demonstrate that short-term treatment with UAG, but not AG, of bone marrow cells in vitro leads to Akt and eNOS phosphorylation and that, in vivo, these events are associated with MMP9 activation and EPC mobilization. Furthermore, UAG, unlike AG, strongly induced EPC mobilization in individuals with type 2 diabetes, but not in nondiabetic subjects. Notably, no change in serum concentrations of primary mobilization factors was detected after UAG or AG systemic administration.

In addition, our results shed light on the earliest molecular events leading to UAG- but not AG-mediated physiological regulation of EPC bioavailability. Indeed, we showed that EPCs possess specific UAG-binding sites, which are not recognized by AG. In addition to GHS-R1a, which is the AG-specific receptor (3), other ghrelin receptor subtypes exist, whose molecular identities have not yet been characterized, but that recognize both AG and UAG (3). Thus, our data provide the first evidence of the existence of UAG-specific binding sites and of their relevance in human-derived EPCs, which could represent a novel target for pharmacological modulation.

Preclinical and clinical studies generally support the therapeutic potential of autologous EPCs in the treatment of cardiovascular diseases, such as tissue ischemia and myocardial infarction (49). However, EPC mobilization may also accelerate atherosclerotic plaque progression (50) and induce tumor (51) or retina (52) neovascularization in individuals with type 2 diabetes. Nonetheless, therapies with statins, the main EPC mobilization mediators (53), revealed no concerns in terms of neovascularization. We now have reason to believe that the significant EPC mobilization potential of UAG, and the lack of its effects on serum levels of SDF-1 and VEGF, may be exploited for clinical treatment of diabetes- and atherosclerosis-induced vascular impairment.

In the presence of cardiovascular risk factors such as diabetes, EPC availability is reduced, restricting the possibility of treating patients in need with directed/cell-based therapies. Besides displaying a positive influence on β-cell viability and glucose homeostasis (3), UAG mobilizes EPCs, protects EPCs from oxidative stress and from senescence, and increases de novo vessel formation (see model in Fig. 7). This suggests that UAG-related peptides or UAG receptor–specific agonists may be further developed into lead compounds from the perspective of a novel pharmacologic intervention to ameliorate both metabolic control and impaired vascular growth in individuals with type 2 diabetes where AG has failed.

ACKNOWLEDGMENTS

M.F.B. was supported by grants from the Italian Association for Cancer Research (AIRC) and Ricerca Finalizzata Regione Piemonte. M.F.B., L.P., and E.G. were supported by MIUR (Ministero dell'Università e Ricerca Scientifica, cofinanziamento MURST and fondi ex-60%).

No potential conflicts of interest relevant to this article were reported.

We thank Dr. Silvia Soddu for kindly providing the vector pSUPER retro containing p53 siRNA and Dr. Silvia Giordano for kindly providing the V12N17Rac construct as well as for fruitful discussion. We thank Dr. Terence Edgar Hébert for his invaluable contribution to final editing of the article.

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