OBJECTIVE—RAGE interacts with the endogenous ligands S100 calgranulins and high mobility group box 1 (HMGB1) to induce inflammation. Since hyperglycemia-induced reactive oxygen species (ROS) activate many pathways of diabetic tissue damage, the effect of these ROS on RAGE and RAGE ligand expression was evaluated.

RESEARCH DESIGN AND METHODS—Expression of RAGE, S100A8, S100A12, and HMGB1 was evaluated in human aortic endothelial cells (HAECs) incubated in normal glucose, high glucose, and high glucose after overexpression of either uncoupling protein 1 (UCP1), superoxide dismutase 2 (SOD2), or glyoxalase 1 (GLO1). Expression was also evaluated in normal glucose after knockdown of GLO1. Expression was next evaluated in high glucose after knockdown of nuclear factor (NF)-κB p65 (RAGE) and after knockdown of activated protein-1 (AP-1) (S100A8, S100A12, and HMGB1), and chromatin immunoprecipitation (ChIP) was performed ± GLO1 overexpression for NFκB p65 (RAGE promoter) and AP-1 (S100A8, S100A12, and HMGB1 promoters). Finally, endothelial cells from nondiabetic mice, STZ diabetic mice, and STZ diabetic mice treated with the superoxidedismutase mimetic Mn(III)tetraphenylporphyrin chloride (MnTBAP) were evaluated.

RESULTS—High glucose increased RAGE, S100A8, S100A12, and HMGB1 expression, which was normalized by overexpression of UCP1, SOD2, or GLO1. GLO1 knockdown mimicked the effect of high glucose, and in high glucose, overexpression of GLO1 normalized increased binding of NFκB p65 and AP-1. Diabetes increased RAGE, S100A8, and HMGB1 expression, and MnTBAP treatment normalized this.

CONCLUSIONS—These results show that hyperglycemia-induced ROS production increases expression of RAGE and RAGE ligands. This effect is mediated by ROS-induced methylglyoxal, the major substrate of glyoxalase 1. Diabetes 59:249–255, 2010

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Hyperglycemia-Induced Reactive Oxygen Species Increase Expression of the Receptor for Advanced Glycation End Products (RAGE) and RAGE Ligands

Dachun Yao and Michael Brownlee

T he receptor for advanced glycation end products (RAGE) is a pattern recognition receptor that interacts with a number of endogenous ligands in normal physiology, playing a homeostatic role in lung development, osteoclast differentiation, innate immunity, and inflammatory cell recruitment and adhesion (1–3). However, conditions such as diabetes disturb homeostasis, increase RAGE expression (4), increase advanced glycation end product formation, and cause release of intracellular calcium binding molecules, the S100 calgranulins (5–7), and the DNA binding protein amphoterin, or high mobility group box 1 (HMGB1), which act as danger signals, called alarmins, that bind to RAGE with high affinity and activate immune cells and vascular endothelium (1,8,9). RAGE signaling stimulates a host of proinflammatory events (1,3). Normally, these appear to play an important role in acute inflammation. In contrast, when responding to persistent elevations of endogenous ligands, RAGE signaling promotes chronic inflammation. Such chronic inflammation plays a major role in the development of diabetic complications, including atherosclerosis (10–12).

Because hyperglycemia-induced reactive oxygen species (ROS) activate many pathways of diabetic tissue damage, including intracellular AGE formation (13,14), the effect of these ROS on RAGE and RAGE ligand expression was evaluated. Although a large number of S100 proteins have been shown to interact with RAGE in cell-based assays (15), S100A8 and S100A12 were selected for study because these proteins are found in high concentrations in inflamed tissue, and they exhibit proinflammatory effects in vitro at concentrations found at sites of inflammation in vivo (9).

RESEARCH DESIGN AND METHODS

Primary human aortic endothelial cells (HAECs) (from Cambrex) and conditionally immortalized HAECs (generated by Dr. Anita Samuga, Albert Einstein College of Medicine) were maintained in EBM-2 medium (from Lonza) with all the supplements. Immortalized HAECs were grown at 33°C, but experiments and treatment were performed at the nonpermissive temperature of 37°C. Mn(III)tetakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from Calbiochem (San Diego, CA). UCP1, SOD2, and GLO1 cDNAs (obtained from Open Biosystems) were cloned into the shuttle vector pAd5CMVK-NpA, and adenoviral vectors and empty control virus were prepared by the Gene Transfer Vector Core at the University of Iowa. Extracellular HMGB1 level and adenoviral vectors and empty control virus were prepared by the Gene Transfer Vector Core at the University of Iowa. Extracellular HMGB1 level was analyzed by an HMGB1 ELISA detection kit (#APO-54N-043 from Apo-tech) according to the manufacturer’s instructions. shRNA lentivirus for human GLO1 and nontarget control were obtained from Sigma. GLO1 mouse antibody was obtained from Abnova. siRNA for the nuclear factor (NF)-κB p65 subunit, activated protein-1 (AP-1) (c-Jun), and scrambled oligonucleotide as control (sc-37007) were obtained from Santa Cruz Biotech. The antibodies for RAGE (sc-74473), S100A8 (sc-20174 for human, and sc-8113 for mouse), HMGB1 (sc-56698), and 3-nitrotyrosine (sc-32731) were obtained from Santa
ROS INCREASE RAGE AND RAGE LIGAND EXPRESSION

Cruz Biotech. Methylglyoxal-modified protein was measured by Western blotting using a monoclonal antibody to the major intracellular methylglyoxal-derivative epitope, N-acetyl-N-(5-hydro-5-methyl)-l-imidazolone.

Measurement of ROS generation. Treated cells seeded in a 96-well plate were incubated with 10 μmol/l CM-H2DCFDA (Invitrogen) for 45 min at 37°C, and the intracellular formation of ROS was measured at excitation/emission wavelengths of 485/530 nm using a Wallac 1420 Fluorescent Plate Reader.

RT reaction and real-time quantitative PCR. Total RNA from cells was extracted using the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen), and the RNA was reverse-transcribed with the SuperScript III First Strand Synthesis System (Invitrogen). Real-time quantitative PCR (qPCR) was run on a LightCycler Roche 480 with LightCycler 480 SYBR Green I Master kit (Roche). PCR was performed by denaturing at 95°C for 7 min, followed by 45 cycles of denaturation at 95°C, annealing at 60°C, and extension at 72°C for 10 s, respectively. Results were normalized by β-actin. Primer sequences are shown in Table 1.

Western blotting. Proteins were separated on 10% SDS-PAGE gels, blotted with the indicated primary antibodies, and then simultaneously incubated with the differentially labeled species-specific secondary antibodies, anti-RABBIT IRDye 800CW (green), and anti-MOUSE (goat) ALEXA680 (red). Membranes were scanned and quantitated with the ODYSSEY Infrared Imaging System (LI-COR, Lincoln, NE).

GLO1 activity assay. GLO1 activity was measured by using a spectrophotometric method that monitors the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione (ε240 = 3.37 mmol l−1 cm−1). The reaction was initiated by addition of 3.37 mmol/l GSH and 3.1 mmol/l NADPH. Enzyme catalyzing formation of 1 ε240 = 3.37 mmol l−1 cm−1. The reaction was initiated by addition of 3.37 mmol/l GSH and 3.1 mmol/l NADPH. Enzyme catalyzing formation of 1 -actin. Primer sequences are shown in Table 1.

FIG. 1. Hyperglycemia-induced ROS increase expression of RAGE, S100 calgranulins, and HMGB1. A: Primary HAECs were infected with UCP1, SOD2, GLO1, or empty control adenovirus (CTL). After incubation with either low glucose (LG) (5 mmol/l) or high glucose (HG) (30 mmol/l) for 5 days, mRNA levels were determined by real-time PCR (n = 3). B: Intracellular protein levels of RAGE, S100A8, and HMGB1 were determined by Western blotting (n = 4). Protein sizes were evaluated by standard protein markers, and their sizes were as follows: RAGE (46 kDa), S100A8 (11 kDa), and HMGB1 (25 kDa). C: Levels of secreted HMGB1 were determined in culture medium by enzyme-linked immunosorbent assay (ELISA) (n = 5). Values are shown as means ± SD, *P < 0.05 vs. LG/CTL group. (A high-quality color digital representation of this figure is available in the online issue.)

TABLE 1
Sequences of primers

| Gene       | Target      | Species                  | Forward primer (5′→3′)                  | Reverse primer (5′→3′)                  |
|------------|-------------|--------------------------|-----------------------------------------|-----------------------------------------|
| β-actin    | mRNA        | Human                    | gatgcaggaagatgactgc                     | atactctgctgctgacga                     |
| RAGE       | mRNA        | Human                    | ctacccgatcctgtctacctca                 | cattcaacgcaagctcgaag                   |
| S100A8     | mRNA        | Human                    | tcatgaaaaaggagggcaga                   | tctaatggagtctagcga                     |
| S100A12    | mRNA        | Human                    | ggagcatcctaaagcgcca                    | acatacctacacacgaccaclte               |
| HMGB1      | Promoter/ChiP| Human                    | caaactctgctgctgcttccataa               | acaactctgccacacatcagga                 |
| S100A8     | Promoter/ChiP| Human                    | caggagggacaaatactctct                   | tggcagacacctacgcag                      |
| β-actin    | mRNA        | Mouse                    | tctttgtttagaatagcgtcgt                 | atctctctctctctgtcga                    |
| vWR        | mRNA        | Mouse                    | cctctctcaggactgcaacag                  | tttgtgttagaatagcgtcgt                 |
| α-actin    | mRNA        | Mouse                    | ttctcctctcaggactgcaacag                | tttgtgttagaatagcgtcgt                 |
| RAGE       | mRNA        | Mouse                    | ttctcctctcaggactgcaacag                | ttctcctctcaggactgcaacag                |
| S100A8     | mRNA        | Mouse                    | caactctcaggactgcaacag                  | caactctcaggactgcaacag                  |
| HMGB1      | mRNA        | Mouse                    | ttctcctctcaggactgcaacag                | ttctcctctcaggactgcaacag                |

β-actin: Levels of RAGE, S100A8, and HMGB1 were measured by Western blotting using a monoclonal antibody to the major intracellular methylglyoxal-derivative epitope, N-acetyl-N-(5-hydro-5-methyl)-l-imidazolone.
and the aorta was dissected and snap-frozen in OCT compound. Then, 10-μm sections were cut by microtome and mounted on PEN (polyethylene naphthalate) membrane slides (2.0 μm, Leica). Aortic endothelial cells or smooth muscle cells were isolated by laser capture microdissection for analysis of mRNA expression level by qPCR. All in vivo procedures were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine. Blood glucose values were 158 ± 10 mg/dl for LG/CTL, 391 ± 21 mg/dl for STZ diabetic, and 376 ± 26 mg/dl for MnTBAP-treated STZ mice. Body weights were 29.7 ± 1.7 g (LG/CTL), 26.7 ± 1.8 g (STZ), and 27.1 ± 1.4 g (MnTBAP-treated STZ mice).

**Statistical analysis.** Results are expressed as means ± SD. All experiments were performed at least in triplicate. Data distribution was analyzed, and statistical differences for different treatments were evaluated by ANOVA and the Tukey-Kramer test using SPSS 15 software.

**RESULTS**

In cultured HAECs, mRNA levels of RAGE, S100A8, and S100A12 calgranulins and HMGB1 were increased by high glucose by 2.3-, 2.1-, 3.4-, and 1.7-fold, respectively (Fig. 1A). Each of these high glucose-induced increases was prevented by overexpression of either uncoupling protein-1 (UCP1) or manganese superoxide dismutase (SOD2), both of which prevent hyperglycemia-induced superoxide production by the mitochondrial electron transport chain (13,14). Increased gene expression of RAGE, S100A8, S100A12, and HMGB1 was also prevented by overexpression of the α-oxoaldehyde degrading enzyme glyoxalase 1 (GLO1). The major physiological substrate for GLO1, methylglyoxal, is a highly reactive dicarbonyl that accumulates in endothelial cells and in several other cell types exposed to hyperglycemia, as a consequence of increased mitochondrial superoxide production. Overexpression of UCP1, SOD2, and GLO1 in cells subjected to 5 mmol/l glucose decreased ROS levels by 40% (data not shown).

Protein expression of RAGE, S100A8, and intracellular HMGB1 paralleled mRNA levels (Fig. 1B). High glucose increased levels 1.7-, 1.9-, and 1.5-fold, respectively. Because HMGB1 is released into the extracellular milieu under stressed conditions (17), extracellular HMGB1 protein was also measured in the medium (Fig. 1C). Extracellular HMGB1 increased 2.2-fold in high glucose. This increase was totally normalized by overexpression of either UCP1, or SOD2, or GLO1.

High glucose increased ROS twofold, and this was prevented by overexpression of either UCP1, SOD2, or GLO1.

**FIG. 2.** Overexpression of UCP1, SOD2, and GLO1 prevents hyperglycemia-induced ROS generation and methylglyoxal formation. Primary HAECs were infected with UCP1, SOD2, GLO1, or empty control adenovirus (CTL). After incubation with either low glucose (LG) (5 mmol/l) or high glucose (HG) (30 mmol/l) for 1 or 5 days, the cells were used for analysis. A: ROS formation on day 5. B: Methylglyoxal-modified protein on day 5. C: Representative blots for B. D: GLO1 activity on day 1. E: GLO1 activity on day 5. F: Representative blots for E. G: GLO1 protein on day 1. H: GLO1 protein on day 5. I: Representative blots for H. n = 4. *P < 0.05 vs. LG/CTL group. Values are shown as means ± SD. (A high-quality color digital representation of this figure is available in the online issue.)
Similarly, high glucose increased levels of methylglyoxal-modified intracellular protein, and this was also prevented by overexpression of either UCP1, SOD2, or GLO1 (Fig. 2B). Representative Western blots are shown in Fig. 2C. Endogenous GLO1 activity and protein levels were unchanged after incubation in high glucose for 24 h (Fig. 2D and E). Representative Western blots for Fig. 2E are shown in Fig. 2F. However, after 5 days, hyperglycemia-induced ROS reduced endogenous GLO1 activity (Fig. 2G) and protein levels (Fig. 2H) by 50%. Representative Western blots for 2 h are shown in Fig. 2F.

To confirm that hyperglycemia-induced increases in intracellular methylglyoxal were sufficient to increase expression of RAGE and RAGE ligands, GLO1 was knocked down 80% by shRNA in cells exposed to normal glucose (Fig. 3A and B). When GLO1 was knocked down, mRNA levels for RAGE, S100A8, S100A12, and HMGB1 were increased by 2.3-, 1.9-, 2.4- and 1.7-fold, respectively, levels equivalent to those induced by high glucose (Fig. 3A). In cells incubated in high glucose, GLO1 siRNA further increased expression of RAGE and RAGE ligands. Similarly, RAGE, S100A8, and HMGB1 protein levels increased 2.1-, 1.8-, and 1.6-fold, respectively, when GLO1 was knocked down in cells exposed to normal glucose (Fig. 3B), levels equivalent to those induced by high glucose. No antibody was available for S100A12. GLO1 knockdown also increased intracellular levels of methylglyoxal-modified protein to that caused by high glucose incubation (Fig. 3C). Representative Western blots for Fig. 3C are shown in Fig. 3D.

We next asked how ROS-induced increases in methylglyoxal-modified protein caused increased expression of RAGE and its endogenous ligands. Induction of RAGE expression by AGEs and ligands such as tumor necrosis factor (TNF)-α requires binding of the transcription factor NFκB (p65/p50) to canonical binding sites in the RAGE

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**FIG. 3.** GLO1 knockdown duplicates the effect of hyperglycemia on expression of RAGE, S100 calgranulins, and HMGB1. Conditionally immortalized HAECS were infected with either shGLO1 or nontargeting control (CTL) lentivirus and then incubated in either low glucose (LG) or high glucose (HG) for 5 days. A: mRNA levels were determined by real-time PCR (n = 3). B: Protein levels were determined by Western blotting (n = 4). Protein sizes were evaluated and confirmed by standard protein markers, and their sizes were as follows: RAGE (46 kDa), S100A8 (11 kDa), and HMGB1 (25 kDa). C: Intracellular methylglyoxal-modified proteins were quantitated by Western blotting (n = 4). D: Representative blots for C. Values are shown as means ± SD. *P < 0.05 vs. CTL group. (A high-quality color digital representation of this figure is available in the online issue.)
promoter, while induction by other ligands requires binding of the transcription factor Sp1 (18,19). Because hyperglycemia-induced ROS cause increased expression of p65 (20), the effect of p65 knockdown by siRNA on RAGE expression was determined. Knockdown of NFκB p65 (80%) prevented increased RAGE expression by high glucose (Fig. 4A). GLO1 knockdown increases p65 expression in the absence of hyperglycemia, and GLO1 overexpression prevents high glucose-induced p65 expression (20). Therefore, the effect of GLO1 overexpression on NFκB p65 binding to the RAGE promoter was determined using ChIP. As shown in Fig. 4B, high glucose increased binding of this transcription factor to the RAGE promoter, and GLO1 overexpression prevented this increase.

Finally, expression of RAGE, S100A8, and HMGB1 was evaluated in aortic endothelial cells isolated by laser capture microdissection from nondiabetic mice, STZ diabetic mice, and STZ diabetic mice treated with the superoxide dismutase mimetic MnTBAP (Fig. 5). S100A12 was not measured, since the S100A12 gene is not expressed in rodents (21). To confirm that laser capture microdissection had successfully isolated endothelial cells, mRNA levels of vWF, an endothelial cell–specific marker; β-actin, a smooth muscle cell marker; and GAPDH, a control gene, were measured (Fig. 5A and B). vWF mRNA was greatly enriched in endothelial cells compared with SMCs, whereas SMCs were greatly enriched in α-actin compared with endothelial cells.

Diabetes increased intracellular ROS, as determined by 3-nitrotyrosine levels, and MnTBAP treatment normalized this (Fig. 5C). Representative Western blots for Fig. 5C are
shown in Fig. 5D. Intracellular levels of methylglyoxal-modified protein were similarly increased by diabetes and normalized by MnTBAP treatment (Fig. 5E). Representative Western blots for Fig. 5E are shown in Fig. 5F.

Diabetes increased RAGE mRNA levels by 1.8-fold, S100A8 calgranulin mRNA levels by 1.7-fold, and HMGB1 mRNA levels by 2.4-fold (Fig. 5G). Treatment of diabetic mice with the superoxide dismutase mimetic compound MnTBAP normalized diabetic endothelial cell RAGE, S100A8, and HMGB1 mRNA levels.

**DISCUSSION**

In the present study, we show that hyperglycemia-induced ROS production by the mitochondrial electron transport chain increases expression of RAGE and three high-affinity endogenous RAGE ligands—S100A8 calgranulin, S100A12 calgranulin, and HMGB1—that exhibit proinflammatory effects in vitro at concentrations found at sites of inflammation in vivo (9). This effect is mediated by ROS-induced production of methylglyoxal, the major α-oxoaldehyde substrate of the enzyme glyoxalase 1. This increases binding of NFκB to the RAGE promoter and of AP-1 to the S100A8, S100A12, and HMGB1 promoters. Diabetes also increased expression of RAGE, S100A8, and HMGB1 in aortic endothelial cells in vivo, and treatment of diabetic mice with the superoxide dismutase mimetic MnTBAP normalized each of these increases.

Much evidence supports a unified mechanism of hyperglycemia-induced cellular damage, in which intracellular hyperglycemia develops in target cells of diabetic complications, causing increased mitochondrial production of ROS. The ROS cause strand breaks in nuclear DNA, which activate the enzyme poly(ADP-ribose) polymerase (PARP). PARP then modifies GAPDH, thereby reducing its activity. This decreased GAPDH activity activates the polyol pathway, increases intracellular AGE formation, activates PKC isoforms, increases NFκB p65 transcription and activity, and activates hexosamine pathway flux.
RAGE was originally identified by its ability to bind advanced glycation end products. However, there is currently disagreement about the importance of AGE-modified proteins as agonists of RAGE in vivo, because proteins modified by AGEs to the extent necessary to bind RAGE are unlikely to exist in physiological systems in vivo (22–24). In contrast, S100/calgranulin proteins and high mobility group-1 protein are present at sites of inflammation in vivo at concentrations that activate RAGE (9,15).

While modification of the corepressor mSin3A by the GLO1 substrate methylglyoxal has been shown to mediate hyperglycemia-induced transcription of angiopoietin-2 (25), the specific methylglyoxal-modified protein(s) responsible for hyperglycemia-induced transcription of RAGE and its ligands S100A8 calgranulin, S100A12 calgranulin, and HMG1B remain to be determined. Regardless of the specific proteins modified, however, the data presented here suggest that pharmacologic agents capable of reducing methylglyoxal concentration in cells susceptible to diabetic complications (14) may have a beneficial effect on the damaging consequences of nonphysiologic RAGE activation (3).

ACKNOWLEDGMENTS
This study was funded by a Center Grant from the Juvenile Diabetes Research Foundation and National Institutes of Health Training Grant 5T32HL007675-20.

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

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