TRANSLATIONAL PHYSIOLOGY

AGE/RAGE produces endothelial dysfunction in coronary arterioles in Type 2 diabetic mice

Xue Gao,1* Hanrui Zhang,1* Ann Marie Schmidt,2 and Cuihua Zhang1

1Departments of Internal Medicine, Medical Pharmacology and Physiology, and Nutritional Sciences, Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri; and 2Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, New York

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Gao X, Zhang H, Schmidt AM, Zhang C. AGE/RAGE produces endothelial dysfunction in coronary arterioles in Type 2 diabetic mice. Am J Physiol Heart Circ Physiol 295: H491–H498, 2008. First published June 6, 2008; doi:10.1152/ajpheart.00464.2008.—We hypothesized that impaired nitric oxide (NO)-dependent dilation (endothelial dysfunction) in Type 2 diabetes results, in part, from elevated production of superoxide (O2•−) induced by the interaction of advanced glycation end products (AGE)/receptor for AGE (RAGE) and TNF-α signaling. We assessed the role of AGE/RAGE and TNF-α signaling in endothelial dysfunction in Type 2 diabetic (Leprdb) mice by evaluating of endothelial function in isolated coronary resistance vessels of normal control (nondiabetic, m Leprdb) and diabetic mice. Although dilation of vessels to the endothelium-independent vasodilator sodium nitroprusside (SNP) was not different between diabetic and control mice, dilation to the endothelium-dependent agonist acetylcholine (ACH) was reduced in diabetic vs. control mice. The activation of RAGE with RAGE agonist S100b eliminated SNP-potentiated dilation to ACh in Leprdb mice. Administration of a soluble form of RAGE (sRAGE) partially restored dilation in diabetic mice but did not affect dilation in control mice. The expression of RAGE in coronary arterioles was markedly increased in diabetic vs. control mice. We also observed in diabetic mice that augmented RAGE signaling augmented expression of TNF-α, because this increase was attenuated by sRAGE or NF-κB inhibitor MG132. Protein and mRNA expression of NAD(P)H oxidase subunits including NOX-2, p22phox, and p40phox increased in diabetic compared with control mice. sRAGE significantly inhibited the expression of NAD(P)H oxidase in diabetic mice. These results indicate that AGE/RAGE signaling plays a pivotal role in regulating the production/expression of TNF-α, oxidative stress, and endothelial dysfunction in Type 2 diabetes.

coronary microcirculation; nitric oxide; reactive oxygen species

A HALLMARK OF DIABETIC VASCULOPATHY, which is postulated to lead to vascular disease, is abrogated endothelial dilation. We reported previously (10, 19, 29) that the inflammatory cytokine tumor necrosis factor-α (TNF-α) plays a pivotal role in endothelial dysfunction; the neutralizing antibody to TNF-α restored blunted dilation in diabetic rats (19), and diabetic mice null for TNF-α did not show endothelial dysfunction (10). TNF-α also affects intracellular insulin signaling in fat, skeletal muscle, and other insulin-sensitive tissues by inhibiting kinase activity in the proximal part of the insulin signaling pathway (20). We found that TNF-α contributes to oxidative stress in diabetes, which may explain, in part, elevations in oxidative stress in diabetes. However, there are other possible contributors to the increased oxidative stress in diabetes, which could serve to amplify the effects of TNF-α. Advanced glycation end products (AGE) and receptor for AGE (RAGE) signaling stimulates the production of superoxide (O2•−), which could further both the oxidative stress and the impaired bioavailability of nitric oxide (NO). In humans with diabetes, the increase in circulating AGE has been found to parallel the severity of diabetic kidney disease. AGE accumulate more quickly than normal in the blood and arteries of patients with diabetes (22). Diabetic rats treated with amnoguanidine (Pimagedine; prevents AGE formation) showed a reversal of inadequate blood flow to the nerves (13) and gradual improvement of the nerves’ ability to transmit signals (27). This suggests that blockade of AGE formation may have potential for treating diabetic neuropathy. Theoretically, as AGE become self-perpetuating and well-established in certain tissues, and even if blood sugar is returned to normal, the AGE might continue to increase, thereby leading to diabetic complications. Recent evidence suggests that inflammation plays a role in the development of insulin resistance and is a predictor of the development of Type 2 diabetes mellitus (2).

Therefore, we proposed that AGE/RAGE contributes to endothelial dysfunction both directly and by regulating the production and expression of TNF-α in Type 2 diabetes. The latter proposition is based on observations showing that nuclear factor-κB (NF-κB), a transcription factor activated by inflammation and oxidative stress, plays a key role in TNF-α expression. Accordingly, we evaluated the expression of AGE/RAGE and TNF-α in coronary arterioles in Type 2 diabetic and normal control (nondiabetic) mice and determined whether AGE/RAGE signaling would compromise endothelial dilation and produce reactive oxygen species (ROS). We also tested whether AGE/RAGE signaling leads to TNF-α expression and production in diabetes.

MATERIALS AND METHODS

Animals. The procedures followed were approved by the Laboratory Animal Care Committee at University of Missouri, Columbia.

* X. Gao and H. Zhang contributed equally to this work.

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Heterozygote controls (m Lepr<sup>db</sup>), wild-type (WT) controls, homozygote Type 2 diabetes (Lepr<sup>db</sup>, diabetic), and Lepr<sup>db</sup> null for TNF-α (dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup>) mice were purchased from Jackson Laboratory and maintained on a normal rodent chow diet. Our studies utilized 12- to 16-wk-old 15- to 25-g m Lepr<sup>db</sup> and WT mice and 25- to 50-g Lepr<sup>db</sup> and dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup> mice of either sex. We used the same strain (C57BL/6J) of m Lepr<sup>db</sup> and dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup> mice to match the backgrounds of Lepr<sup>db</sup> mice. The dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup> mice show the phenotype of hyperglycemia and obesity, the diabetic phenotype that is consistent with the penetrance of the leptin receptor mutation. The obese mice from the second round of breeding of Lepr<sup>db</sup> and TNF-α/- were used in experimentation. We defined m Lepr<sup>db</sup> and WT mice as controls in this study because the results from m Lepr<sup>db</sup> and WT mice were identical. The Type 2 diabetic Lepr<sup>db</sup> mouse is designated as the diabetic mouse in this study.

Measurement of metabolic parameters. The methods for measuring blood glucose, lipid level, and blood pressure (BP) were described in detail previously (10), and hemoglobin A1c (HbA1c) level, the index of glycosylated hemoglobin, is consistent with the penetrance of the leptin receptor mutation. The blood glucose, lipid level, and blood pressure (BP) were described in detail previously (10), and hemoglobin A1c (HbA1c) level, the index of glycosylated hemoglobin, is consistent with the penetrance of the leptin receptor mutation.
Table 1. Baseline metabolic parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic Anti-TNF</th>
<th>dbTNF+/dbTNF−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>25 ± 7</td>
<td>49 ± 5*</td>
<td>50 ± 8*</td>
<td>47 ± 7*</td>
</tr>
<tr>
<td>BP, mmHg</td>
<td>116 ± 13</td>
<td>121 ± 12</td>
<td>119 ± 11</td>
<td>126 ± 17</td>
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<tr>
<td>Abdominal girth, cm</td>
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<td>15 ± 4*</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
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<td>156 ± 8*</td>
<td>153 ± 5*</td>
<td>146 ± 7*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>56 ± 3</td>
<td>89 ± 2</td>
<td>93 ± 5</td>
<td>97 ± 4</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>62 ± 8</td>
<td>96 ± 13*</td>
<td>97 ± 10*</td>
<td>90 ± 8*</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>137 ± 19</td>
<td>329 ± 21*</td>
<td>327 ± 20*</td>
<td>325 ± 27*</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
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<td>13 ± 4*</td>
<td>10 ± 3*</td>
<td>11 ± 2*</td>
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<tr>
<td>Blood HbA1c, %</td>
<td>4.6 ± 0.3</td>
<td>7.8 ± 0.6*</td>
<td>5.2 ± 0.5†</td>
<td>5.0 ± 0.4†‡</td>
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Values are means ± SE hemodynamic and metabolic parameters in control mice, diabetic mice, diabetic mice treated with anti-TNF, and dbTNF+/dbTNF− mice (n = 10). BP, blood pressure; HbA1c, hemoglobin A1c. *P < 0.05 vs. control; †P < 0.05 vs. diabetic; §P < 0.05 vs. diabetic in each group.

Serum AGE production and mRNA expression of RAGE. Serum AGE production (Fig. 1A) in diabetic mice was significantly increased compared with control mice, but aminoguanidine (inhibitor of AGE formation) greatly attenuated serum AGE production in diabetic mice without affecting serum AGE production in control mice. Normalization of RAGE transcripts to those of β-actin demonstrated significantly greater (~9-fold) mRNA expression of RAGE in the coronary arterioles of diabetic mice than in control mice. Moreover, aminoguanidine attenuated RAGE expression in diabetic mice but did not affect that in control mice (Fig. 1B). Serum AGE production and mRNA expression of RAGE are significantly attenuated in dbTNF+/dbTNF− mice compared with diabetic mice (Fig. 1).

Role of RAGE in ACh-induced NO-mediated vasodilation. Figure 2A shows responses to ACh in control mice after incubation of the vessels with SNP (0.1 μM) and after incubation of the vessels with S100b in the presence of SNP (0.1 μM). Figure 2B shows responses to ACh in diabetic mice after incubation of the vessels with S100b (0.1 μM) and after incubation of the vessels with S100b in the presence of SNP (0.1 μM). We were expecting a parallel shift in the line due to the constant amount of SNP, but the amplification of dilation was unexpected. Nonetheless, activation of RAGE with S100b eliminated this effect. **Table 1. Baseline metabolic parameters**

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**Fig. 1.** A: serum advanced glycation end products (AGE) production in control, diabetic, and dbTNF+/dbTNF− mice and control and diabetic mice treated with aminoguanidine (AGD). B: expression of receptor for AGE (RAGE) mRNA in isolated coronary arterioles of control and diabetic mice, control and diabetic mice treated with AGD, and dbTNF+/dbTNF− mice. Data are means ± SD; n = 8. *P < 0.05 vs. control, #P < 0.05 vs. diabetic in each group.

**Fig. 2.** A: ACh-induced vasodilation in control mice after incubation with sodium nitroprusside (SNP) alone or both SNP and S100b was identical to control (n = 6). B: vasodilation to ACh was potentiated in diabetic mice after incubation with SNP (diabetic vs. diabetic + SNP), but this SNP-potentiated dilation to ACh was abolished after incubation with both SNP and S100b (diabetic + SNP vs. diabetic + SNP + S100b; n = 9). *P < 0.05 vs. diabetic, #P < 0.05 vs. diabetic + SNP + S100b.
Role of RAGE in endothelial dysfunction and NAD(P)H oxidase activity in Type 2 diabetes. Coronary arterioles isolated from control and diabetic mice dilated in a concentration-dependent manner to ACh, although dilation was reduced in diabetic mice (Fig. 3A). However, the impaired vasodilation in diabetic mice was improved by 50% after corruption of AGE/RAGE signaling with sRAGE (Fig. 3A). Figure 3B shows NAD(P)H oxidase activity from isolated coronary arterioles examined in control, diabetic, and dbTNF−/dbTNF− mice and diabetic mice treated with sRAGE or MG132. sRAGE and NF-κB inhibitor MG132 decreased NAD(P)H oxidase activity in diabetic mice.

Protein expression of RAGE in isolated coronary arterioles. The protein expression of RAGE (Fig. 4) was higher in diabetic mice than in control mice, but RAGE expression in TEMPOL-, anti-TNF-, sRAGE-, or MG132-treated diabetic mice was similar compared with that in control mice. sRAGE or MG132 did not affect the protein expression of RAGE in control mice (Fig. 4B). The protein expression of RAGE was greatly attenuated in dbTNF−/dbTNF− mice compared with diabetic mice (Fig. 4B).

Protein expression of TNF-α and NF-κB in isolated coronary arterioles. TNF-α expression (Fig. 5, A and B) was elevated over twofold and NF-κB expression (Fig. 5C) was elevated about fourfold in diabetic mice. sRAGE or MG132 decreased TNF-α and NF-κB expression in diabetic mice but did not affect expression in control mice. NF-κB expression was similar in dbTNF−/dbTNF− mice and control mice.

mRNA and protein expression of NAD(P)H oxidase subunits. mRNA expression (Fig. 6) for NAD(P)H oxidase (NOX-2, p22phox, and p40phox) was higher in diabetic mice than in control mice; mRNA expression of p47phox, p67phox, NOX-1, and NOX-4 (data not shown) was similar in diabetic and control mice; and mRNA expression of NAD(P)H oxidase subunits was greatly attenuated in dbTNF−/dbTNF− mice compared with diabetic mice. Anti-TNF or sRAGE or MG132 significantly attenuated the expression of NAD(P)H oxidase in diabetic mice. Moreover, the results of protein expression for NAD(P)H oxidase (NOX-2, p22phox, and p40phox) were identical to the mRNA expression in
isolated coronary arterioles in control, diabetic, and dbTNF−/− mice (data not shown).

DISCUSSION

Our major findings are that overproduction/expression of AGE and RAGE contributes to endothelial dysfunction in Type 2 diabetes. Our findings support the concept that AGE/RAGE and TNF-α signaling is key to the production of ROS; then AGE/RAGE and TNF-α signaling interact to amplify the oxidative stress and induce endothelial dysfunction in diabetic mice.

Role of TNF-α and AGE/RAGE signaling in Type 2 diabetes. The basis for AGE being involved in the pathophysiological sequelae of vascular dysfunction in Type 2 diabetes stems from hyperglycemia that contributes to the production of AGE. Hyperglycemia and oxidant stress promote nonenzymatic glycoxidation of proteins and lipids (7). AGE-mediated generation of low levels of ROS can result in quenching of the endogenous vasorelaxant NO (7). Activation of RAGE by its various ligands reportedly induces a variety of proinflammatory and procoagulant cellular responses, resulting from the activation of NF-κB, including the expression of vascular cell adhesion molecule-1 (VCAM-1), TNF-α, IL-6, and tissue factor (TF) (24). The interaction of AGE with RAGE induces the produc-

![Graph](image_url)

Fig. 5. Western blot analysis for TNF-α protein expression (A and B) and NF-κB protein expression (C). Bars represent the increased expression in diabetic mice as fold change of control mice, e.g., 2 represents a doubling of expression. Data are means ± SD; n = 4. *P < 0.05 vs. control mice, #P < 0.05 vs. diabetic mice.

![Graph](image_url)

Fig. 6. Real-time PCR analysis for NADPH oxidase subunit [NOX-2 (gp91phox, A), p22phox (B), p40phox (C)] mRNA expression (expressed as % of control) from coronary arterioles in control, diabetic, and dbTNF−/− mice and diabetic mice treated with sRAGE or MG132. Data are means ± SD; n = 5. *P < 0.05 vs. control mice, #P < 0.05 vs. diabetic mice.
tion of ROS, which can stimulate the cascade leading to NF-κB-induced transcriptional events. NF-κB will induce expression of TNF-α (5, 23). Accordingly, we determined whether AGE/RAGE signaling leads to TNF-α expression and production in diabetes. Our results showed that protein expression of RAGE, TNF-α, and NF-κB was elevated in Type 2 diabetes. In Type 2 diabetic mice, neutralizing antibody to TNF-α, the free radical scavenger TEMPOL, sRAGE (to corrupt AGE/RAGE signaling), or the NF-κB inhibitor MG132 decreased the protein expression of RAGE, but sRAGE and MG132 also attenuated TNF-α expression. These data provided the evidence for the interaction of AGE/RAGE with TNF-α, which may then stimulate the production of O₂•⁻ via NF-κB in Type 2 diabetes.

Moreover, our results showed that vasodilation to ACh was potentiated in diabetic mice after incubation with SNP, but SNP-potentiated dilation to ACh was abolished after incubation with both SNP and the RAGE agonist S100b. These provocative results further support our idea that AGE/RAGE signaling plays a role in endothelial dysfunction in diabetes. We found the fact that the treatment with SNP potentiated the response to ACh in diabetic arterioles puzzling and have only speculation that is unconfirmed. It is possible that administration of SNP, and the subsequent donation of NO, helps the vascular cells scavenge excess superoxide, thus shifting them into a more favorable redox balance. The generation of AGE and augmentation of proinflammatory mechanisms in the vessel, at least in part via accumulation of S100/calgranulins and amphoterin released from activated inflammatory cells, provides a potent feedback loop for sustained oxidant stress, thereby contributing to the observed endothelial dysfunction.

AGE/RAGE signaling plays a pivotal role in regulation of TNF-α expression. Al-though this does not address any particular risk factor directly, the formation of AGE is known to occur in diabetes, and the oxidative stress induced by AGE/RAGE signaling activates NF-κB. AGE can also reduce the bioavailability and activity of endothelium-derived NO. Serum AGE in patients with Type 2 diabetes is inversely related to the degree of endothelium-dependent and endothelium-independent vasodilation in the brachial artery (22). Aminoguanidine decreases vascular AGE

Fig. 7. Schematic figure showing the interactions among TNF-α, AGE/RAGE, and NF-κB signaling. In brief, central to the endothelial dysfunction is oxidative stress. The oxidative stress is induced by the production of reactive oxygen species (ROS), and this induces NF-κB activation. Key to the production of ROS is AGE/RAGE and TNF-α signaling. We have shown interactions among oxidative stress, AGE/RAGE, and TNF-α because oxidative stress induces NF-κB, this transcription factor can induce both RAGE and TNF-α expression, and TNF-α can induce RAGE expression. Thus the oxidative stress of diabetes begets more oxidative stress, eventually inducing endothelial dysfunction, because of decreased bioavailability of nitric oxide (NO) (due to the reaction between NO and O₂•⁻). EC, endothelial cells; VSMCs, vascular smooth muscle cells; TNFR, TNF-α receptor.
accumulation and severity of atherosclerotic plaque in diabetic rats (8). In our study, vasodilation to the endothelium-independent vasodilator SNP was identical in control and diabetic mice, and dilation to the endothelium-dependent agonist ACh was reduced in diabetic vs. control mice. These results are consistent with our previous study (10). Blocking the formation of AGE or interaction with RAGE are obvious targets for therapeutics. The studies to address this were conducted with the inhibitor of AGE/RAGE formation, signaling, and interactions, sRAGE, to block the RAGE signaling pathway.

In diabetic ApoE-null mice, in which vascular lesions were already established, treatment with sRAGE induced regression of atherosclerosis and lesion complexity (the percentage of complex lesions decreased as they were converted to fatty streaks) (18). This finding is consistent with ours, in which impaired vasodilation was partially restored by administration of sRAGE, because preservation of endothelial function appears to be anti atherosclerotic. Furthermore, sRAGE decreased TNF-α protein expression in diabetic mice (10). Our results support the concept that the interaction of TNF-α with AGE/RAGE contributes to endothelial dysfunction in diabetic mice. To our knowledge, this is the first study to link the mechanism of coronary arteriolar endothelial dysfunction with AGE/RAGE signaling in Type 2 diabetes.

**AGE/RAGE and ROS in Type 2 diabetes.** AGE that bind to RAGE on the endothelial cell surface can lead to a signaling cascade, stimulating NAD(P)H oxidase and increasing the production of ROS (25, 28). The key target of RAGE signaling is NF-κB causing pathological changes in gene expression (3, 25, 28). AGE also may decrease NO availability by decreasing nitric oxide synthase (NOS) activity (4). In our previous study (10), we confirmed the links among TNF-α, NAD(P)H oxidase, ROS, and impaired vasodilation in coronary arterioles in Type 2 diabetes. This study provides further experimental evidence for an interactive signaling pathway of AGE/RAGE and TNF-α. AGE/RAGE appear to sum with TNF-α to induce the endothelial dysfunction in Type 2 diabetes. A previous report showed AGE production increased in diabetic retinal vessels (21) and renal glomeruli (11). High expression of AGE, RAGE, and NF-κB in lacrimal glands of diabetic rats (1) suggests that these factors are involved in signaling and in subsequent inflammatory alterations related to diabetes mellitus. RAGE has been implicated in the pathogenesis of diabetic complications. AGE products and RAGE signaling induces oxidative stress and leads to activation of the transcription factor NF-κB (5, 7). Because TNF-α has four NF-κB sites in its promoter, we postulate that AGE/RAGE signaling increases TNF-α expression.

Our results showed that the free radical scavenger TEMPOL attenuated the protein expression of RAGE in Type 2 diabetes, which suggests a link between RAGE and superoxide. NAD(P)H oxidase inhibitor (apocynin) restored endothelium-dependent dilation in diabetic mice (10). Expression (mRNA and protein) of NAD(P)H oxidase subunits (NOX-2, p22phox, and p40phox) and NAD(P)H oxidase activity were significantly higher in diabetic mice than in control mice. Anti-TNF, sRAGE, or MGI32 significantly inhibited the expression of these NAD(P)H subunits in Type 2 diabetes. Moreover, expression of NAD(P)H oxidase subunits was greatly attenuated in dbTNF/dbTNF− mice compared with diabetic mice, and anti-TNF attenuated the protein expression of RAGE in diabetic mice. These results indicated that the oxidative stress is induced by the production of ROS, which then activates NF-κB. Key to the production of ROS is AGE/RAGE and TNF-α signaling. We have shown interactions among oxidative stress, AGE/RAGE, and TNF-α, because oxidative stress induces NF-κB, and this transcription factor induced both RAGE and TNF-α expression and TNF-α induced RAGE expression (Fig. 7).

In conclusion, our results indicate that the oxidative stress of diabetes begets more oxidative stress, eventually inducing endothelial dysfunction, because of decreased bioavailability of NO. We believe that understanding endothelial dysfunction is critical because the progression of vascular disease may be halted if endothelial dysfunction is rectified. Our study suggests that central to the endothelial dysfunction is oxidative stress; the oxidative stress is induced by the production of ROS, and this induces NF-κB. We envision a scheme in which oxidative stress induced by one of the stimuli begets further oxidative stress by the other, and this becomes the basis for a pathological spiral leading to vascular disease. We believe that TNF-α is further upstream in the hierarchy because TNF-α induces endothelial dysfunction in the prediabetic metabolic syndrome—presumably before AGE/RAGE signaling is pivotal. Our data demonstrated relevance and translation to the cardiovascular disease. Together, these results provide support for our idea that the interaction between TNF-α and RAGE contributes, perhaps even by amplifying one another, toward the evolution of endothelial dysfunction and vascular disease in diabetes. These findings may provide further insight into a novel therapeutic target for cardiovascular diseases associated with TNF-α and AGE/RAGE signaling.

**ACKNOWLEDGMENTS**

The design of Fig. 7 was created by Dr. Xiuping Chen from the laboratory of C. Zhang.

**GRANTS**

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role of age/rage in type 2 diabetes

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