Advanced glycation end products (AGEs) have been implicated in the accelerated vascular injury occurring in diabetes. We recently reported that LDL prepared from type 2 diabetic patients (dm-LDL), but not normal LDL (n-LDL) triggered signal transducers and activators of transcription STAT5 activation and p21^waf expression in endothelial cells (ECs). The aims of the present study were to investigate the role of LDL glycation in dm-LDL–mediated signals and to analyze the molecular mechanisms leading to STAT5 activation. We found that glycated LDL (gly-LDL) triggered STAT5 activation, the formation of a prolactin inducible element (PIE)-binding complex containing STAT5, and increased p21^waf expression through the activation of the receptor for AGE (RAGE). We also demonstrated that dm-LDL and gly-LDL, but not n-LDL treatment induced the formation of a stable complex containing the activated STAT5 and RAGE. Moreover, gly-LDL triggered src but not JAK2 kinase activity. Pretreatment with the src kinase inhibitor PP1 abrogated both STAT5 activation and the expression of p21^waf induced by gly-LDL. Consistently, gly-LDL failed to activate STAT5 in src^-/- fibroblasts. Collectively, our results provide evidence for the role of glycation in dm-LDL–mediated effects and for a specific role of src kinase in STAT5-dependent p21^waf expression. Diabetes 51: 3311–3317, 2002

The development of vascular complications is common to diabetes, and qualitative LDL abnormalities are generally recognized to be closely associated (1,2). Indeed, LDL glycation and oxidation (3,4), alone or in combination, can account for the increased athrogenic risk in diabetes (5). Moreover, recent observations suggest an emerging role for the products of nonenzymatic glycoxidation of proteins and/or lipids (advanced glycation end products [AGEs]) in the pathogenesis of diabetes complications (6) as well as in the vascular complications of hypercholesterolemic rabbits without diabetes (7).

AGE, by ligation to a specific surface receptor (receptor for AGE [RAGE]), triggers a range of cellular responses mainly dependent on activation of the transcriptional factor nuclear factor (NF)-κB (6). Although the means by which the signals reflecting AGE/RAGE interaction are transmitted to the nucleus are only partially known, several lines of evidence indicate that engagement of RAGE by AGE triggers the activation of well known signaling pathways, such as p21ras, mitogen-activated protein (MAP) kinases and CDC42/rac (6). More recently, it has been shown that the proliferative response of kidney interstitial fibroblasts to AGE depends on the activation of the signal transducers and activators of transcription (STATs) (8). The activation of the latent cytoplasmic protein STATs, upon receptor stimulation, is mediated by protein tyrosine kinases belonging to the JAK family (9) or other nonreceptor tyrosine kinase, such as c-src (10). After tyrosine phosphorylation, STAT proteins acquire DNA-binding activity, translocate into the nucleus, bind to specific promoter elements, and control the expression of target genes (9). Among the STAT proteins, STAT5s are pleiotropic regulators of many genes, and their involvement in pathological conditions is well established (11,12). In particular, a role of STAT5 as regulator of gene expression in diabetes-associated vascular disease is suggested by the demonstration that LDL recovered from type 2 diabetic patients (dm-LDL) induced a STAT5-dependent p21^waf expression and inhibition of cell-cycle progression in endothelial cells (ECs) (13).

The aims of the present study were to characterize the upstream effectors involved in dm-LDL–mediated STAT5 activation and p21^waf expression and to evaluate whether LDL glycation may account for the dm-LDL–mediated effects.

RESEARCH DESIGN AND METHODS

Reagents. M199 medium (endotoxin tested), BSA, and human glycated albumin (AGE) were from Sigma Chemical (St. Louis, MO). Nitrocellulose filters, horseradish peroxidase–conjugated protein A, molecular weight markers, chemiluminescence reagent, and [γ^32P]-ATP were from Amersham (Braunschweig, Germany). Endotoxin contamination in LDL preparation was tested by the Limulus amebocyte assay, and the concentration was <0.1 ng/ml. PP1 and AG-490 were from Calbiochem (San Diego, CA). Antiserum. Phospho-p44/42 MAP kinase, phospho-STAT5, and monoclonal anti-phosphotyrosine were from New England BioLabs (Beverly, MA). Peroxidase–conjugate goat anti-mouse IgG, anti–STAT5 and anti–RAGE antiserum, RAGE-blocking peptide, monoclonal anti–integrin adhesion molecule (ICAM), anti–vascular cell adhesion molecule (VCAM), and anti- src antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany).
Patients and control subjects. Blood was withdrawn from type 2 diabetic patients. None of the diabetic patients was taking insulin, and all were treated with sulfonylureas. Nine healthy subjects with normal blood analysis were used as control subjects. Mean age was 68 ± 7 and 65 ± 5 years for diabetic and control subjects, respectively.

Isolation, characterization of LDL. Blood was collected from normal and diabetic subjects and processed as previously described (13). LDLs were separated according to Redgrave and Carlson (14). Capillary electrophoresis was performed as described (15,16). After capillary electrophoresis, the samples were pooled. Glycerated-LDL percentage was evaluated by affinity chromatography using phenyl-boronic agarose (Sigma), as described (17). Two peaks of LDL, corresponding to glycerated and nonglycerated LDL were detected at 254 nm, and their corresponding peak area was integrated.

In vitro LDL glycation. Plasma and whole blood recovered from the same donor were incubated with 0.3 mmol/l EDTA (pH 7.4) at 37°C for 6 weeks in the absence or presence of freshly prepared 25 mmol/l glucose. After 6 weeks, glycation was evaluated on whole blood sample by assessing the degree of HbA1c. HbA1c raised from 4.5% (blank sample) to 17.2% (in vitro glycerated samples). Gly-LDL was isolated and characterized as described above.

Cell culture and transfection. ECs were isolated from human umbilical cord vein (HUCV) and were cultured and characterized as previously described (18). Wild-type and src−/− fibroblasts were cultured as described (19).

Immunoprecipitation, SDS-PAGE, and immunoblotting. HUCV monolayer was serum-starved in M199 containing 1% BSA, 30% PBS (v/v), 0.2 mmol/l sodium orthovanadate, and 1 mmol/l EDTA for 4 h at 37°C, incubated without or with the indicated stimuli, and processed as previously described (20).

Preparation of nuclear extract and gel retardation assay. Nuclear extracts from unstimulated or gly-LDL-treated HUCV were prepared as described by Sadowski and Gilman (21). The oligonucleotides used correspond to the proline inducible element (PIE) (22). Gel retardation reactions were performed as previously described (13,20).

Src kinase assay. Src immunoprecipitated from unstimulated or gly-LDL-stimulated cells were divided into two aliquots. Src immunoprecipitates were resuspended in 50 μl kinase buffer supplemented with 5 μC of γ32P ATP, 1 mmol/l ATP, and 5 μg acid-denatured endonuclease. Phosphorylation of an anti-RAGE antiserum to STAT5 was measured and found to correspond to the HbA1c percentage. Pools of LDL with the same glycation was measured and found to correspond to the HbA1c percentage. Pools of LDL with the same percentage of HbA1c were used.

RESULTS

LDL preparation and characterization. In all diabetic patients, the peak of cholesterol was found in the LDL subfractions with a density ranging from 1.037 to 1.044 g/ml. In all diabetic samples, LDLs were consistently small and dense (13), and the characteristic low cholesterol–apoB ratio was found in these subjects (Table 1). After characterization of all samples, pilot experiments were performed using single preparations; in subsequent experiments, pooled sera from patients was used.

Gly-LDL triggers STAT5 activation and the formation of a PIE binding complex. We have previously reported that dm-LDL was able to induce STAT5 activation (13). To evaluate the role of glycation in mediating such a biochemical event, LDL obtained from normal subjects was glycerated in vitro and assayed. As shown in Fig. 1A, gly-LDL, but not normal LDL (n-LDL), was able to induce STAT5 tyrosine phosphorylation. Consistent with the role of STAT5 in regulating gene transcription, nuclear extracts from gly-LDL-stimulated but not from untreated cells were able to induce the formation of a DNA-protein complex using the PIE sequence, which is the canonical STAT5 binding region (Fig. 1B). The presence of STAT5 in the DNA-protein complex induced by gly-LDL was demonstrated by the ability of the antibody to STAT5 to supershift the PIE-binding complex (Fig. 1B). Similar results were obtained using the p21SIE2 as a probe (data not shown).

TABLE 1
Characteristics of LDL and HbA1c.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Cholesterol (μg/μl)</th>
<th>ApoB (μg/μl)</th>
<th>Cholesterol/ApoB</th>
<th>HbA1c (%)</th>
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</thead>
<tbody>
<tr>
<td>1 (d)</td>
<td>1.61</td>
<td>1.26</td>
<td>1.3</td>
<td>12</td>
</tr>
<tr>
<td>2 (d)*</td>
<td>0.65</td>
<td>0.50</td>
<td>1.3</td>
<td>11.8</td>
</tr>
<tr>
<td>3 (d)*</td>
<td>1.50</td>
<td>1.06</td>
<td>1.4</td>
<td>15.8</td>
</tr>
<tr>
<td>4 (d)</td>
<td>1.93</td>
<td>1.41</td>
<td>1.4</td>
<td>13.9</td>
</tr>
<tr>
<td>5 (d)</td>
<td>1.65</td>
<td>1.16</td>
<td>1.4</td>
<td>12.4</td>
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<td>1.3</td>
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<tr>
<td>7 (d)</td>
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<td>0.75</td>
<td>1.4</td>
<td>11.3</td>
</tr>
<tr>
<td>8 (d)</td>
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<td>0.81</td>
<td>1.35</td>
<td>12.3</td>
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<tr>
<td>9 (d)</td>
<td>0.71</td>
<td>0.49</td>
<td>1.4</td>
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</tr>
<tr>
<td>10 (d)†</td>
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<td>1.4</td>
<td>10</td>
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<tr>
<td>11 (d)†</td>
<td>1.25</td>
<td>0.89</td>
<td>1.4</td>
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</tr>
<tr>
<td>12 (d)†</td>
<td>1.51</td>
<td>1.16</td>
<td>1.3</td>
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</tr>
<tr>
<td>13 (d)†</td>
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<td>1.34</td>
<td>1.3</td>
<td>14.5</td>
</tr>
<tr>
<td>14 (d)†</td>
<td>1.44</td>
<td>1.06</td>
<td>1.35</td>
<td>16.3</td>
</tr>
<tr>
<td>15 (d)†</td>
<td>1.35</td>
<td>1.03</td>
<td>1.3</td>
<td>15</td>
</tr>
<tr>
<td>16 (d)†</td>
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<td>0.78</td>
<td>1.4</td>
<td>8</td>
</tr>
<tr>
<td>17 (d)†</td>
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<td>0.90</td>
<td>1.3</td>
<td>8.3</td>
</tr>
<tr>
<td>18 (d)†</td>
<td>1.05</td>
<td>0.80</td>
<td>1.3</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>1.19</td>
<td>0.73</td>
<td>1.6</td>
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</tr>
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<td>0.98</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>25 (n)</td>
<td>1.36</td>
<td>0.85</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>26 (n)*</td>
<td>1.88</td>
<td>1.04</td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td>27 (n)</td>
<td>0.89</td>
<td>0.56</td>
<td>1.6</td>
<td>1</td>
</tr>
</tbody>
</table>

n, LDL from a healthy subject; d, LDL from type 2 diabetic patients. *Samples used for pilot experiments; †Samples tested for gly-LDL shown). The ability of AGE (Sigma Chemical) to induce activation and nuclear translocation of STAT5 was also evaluated. Because the results obtained with AGE and those obtained with gly-LDL were overlapping, we substantially used gly-LDL throughout the experiments.

Moreover, to assess whether STAT5 activation was dependent on the level of LDL glycation, LDL were obtained from diabetic patients with different percentages of HbA1c: 8, 10, and 15% (Table 1). In each patient, the level of LDL glycation was measured and found to correspond to the HbA1c percentage. Pools of LDL with the same glycation levels were tested for their ability to trigger STAT5 activation in ECs. The dose response effect on STAT5 reported in Fig. 1C demonstrated that 8% glycated-LDL was ineffective, whereas both 10, 15, and 50% (data not shown) LDL glycation induced similar levels of STAT5 tyrosine phosphorylation. Similar results were obtained with in vitro gly-LDL, with corresponding percentages of glycation (data not shown).

dm-LDL-mediated Erk1/Erk2 MAP kinase activation and p21war expression depend on RAGE activation. AGE-mediated RAGE engagement triggers the activation of intracellular signaling molecules, including the Erk1/Erk2 MAP kinases (24). RAGE engagement in mediating Erk1/Erk2 MAP kinase activation was thus evaluated in cells pretreated with the blocking anti-RAGE antisera before dm-LDL, n-LDL, or gly-LDL treatment. As shown in Fig. 2A, after RAGE blockade the Erk1/Erk2 MAP kinase activation was strongly reduced in cells stimulated with gly-LDL or dm-LDL, but not in cells treated with n-LDL.
Thus, this data indicates that RAGE may be the receptor engaged by dm-LDL to trigger Erk1/Erk2 MAP kinase activation. We further evaluated whether RAGE engagement might also occur in dm-LDL-induced p21waf expression. To this end, ECs were stimulated with n-LDL, dm-LDL, or gly-LDL in the presence or absence of a blocking anti-RAGE antibody. Evidently, as reported in Fig. 2B, the blockade of RAGE almost completely abrogates the effect of dm-LDL and gly-LDL on p21waf expression.

Moreover, to assess whether glycated proteins could affect cell-cycle machinery in the absence of p21waf, p21−/− fibroblasts (25) were treated with gly-LDL and dm-LDL and tested for the expression of other cell-cycle related proteins, such as p27, cdk2, and cdk4. The results (not shown) failed to reveal changes in the expression of these proteins.

A stable complex containing the activated STAT5 and RAGE was induced by dm-LDL and gly-LDL. To extend the analysis of the molecular mechanism involved in STAT5 activation, we evaluated whether activated STAT5 and RAGE can interact in response to n-LDL, gly-LDL, or dm-LDL. For this purpose, we performed coimmunoprecipitation experiments, and the results reported in Fig. 3 show that RAGE could be immunoprecipitated by the anti-STAT5 antibody in response to both dm- and gly-LDL, but not in response to n-LDL. The specificity of the anti-RAGE antiserum was evaluated by immunoadsorption experiments. As shown in the right panel, after absorption, the coprecipitating bands were undetectable.

Src kinase, but not JAK2, is involved in gly-LDL–induced STAT5 activation. JAK2 is known to be activated by cytokines that signal to STAT5 (10); therefore, we evaluated the effect exerted by gly-LDL or dm-LDL on JAK2 activation. ECs were stimulated for different time periods, but no JAK2 tyrosine phosphorylation was detected (data not shown). Likewise, in the presence of the JAK2 inhibitor AG-490, gly-LDLs were still able to activate STAT5, indicating that other kinases are involved in STAT5 activation (Fig. 4A). Src kinase is known to activate STAT5 in cytokine-mediated signal (26). Thus, we investigate the effect of gly-LDL on src kinase activity and found that, indeed, the kinase activity of src was induced by gly-LDL (Fig. 4B). This observation led us to analyze the effect of the src kinase inhibitor PP1 [4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine] on gly-LDL-mediated STAT5 activation and p21waf expression. As shown in Fig. 4C, the addition of PP1 completely inhibited the effect of gly-LDL on STAT5 activation as well as on p21waf expression. On the contrary, it was unable to affect the expression of cdk4 or other related proteins (data not shown).

To further validate the role of src kinase in gly-LDL–mediated STAT5 activation, wild-type and src−/− fibroblasts were used (19). The cells were unstimulated or stimulated with gly-LDL and assayed for STAT5 activation. The results reported in Fig. 5B demonstrated that gly-LDL was able to trigger STAT5 activation in wild-type fibroblasts. Conversely (Fig. 5A), stimulation of src−/− fibroblasts with gly-LDL failed to trigger STAT5 tyrosine phosphorylation, indicating that, indeed, the src kinase is involved in the gly-LDL–mediated STAT5 signaling pathway.

It is known that RAGE-AGE interaction induces the expression of adhesion molecules such as VCAM-1 (27). To evaluate whether src kinase was also involved in this effect, ECs were stimulated with gly-LDL or dm-LDL in the presence or absence of PP1. As shown in Table 2, gly-LDL and dm-LDL were able to increase the expression of VCAM-1, and pretreatment with PP1 did not prevent this effect. By contrast, consistent with previous reports (27), either gly-LDL or dm-LDL failed to modulate the expression of ICAM-1 in ECs.

DISCUSSION

STAT proteins are transcriptional factors that play key roles in intracellular signaling after stimulation with vari-
More recently it has been shown that, in kidney interstitial fibroblasts, AGEs may activate some members of the STAT family (8). Among the members of the STAT family, STAT5 was originally described as a transcriptional factor recognizing a specific palindromic sequence, which was originally found in the PIE of the β-casein promoter (22). STAT5 consists of two highly homologous genes defined as STAT5A and STAT5B (22) that undergo activation in response to different stimuli and exert transcriptional activation on a number of genes mainly involved in the control of cell proliferation (11). We previously demonstrated that in ECs, dm-LDL was able to induce p21waf expression by activating STAT5 (13). Herein, we demonstrate that, as a response to dm-LDL, STAT5 also becomes activated in response to gly-LDL and, consistent with its ability to bind the PIE sequence, gly-LDL was able to induce the formation of a PIE-binding complex containing STAT5. Glycated proteins are found at low levels in normal individuals during aging; however, significantly higher levels are found in certain disease states, including diabetes. In this regard, AGE level has been shown to mainly depend on a long period of poor metabolic control and correlates with the severity of some diabetes complications (28). We herein demonstrated that glycated lipoproteins generate, via RAGE engagement, a transcriptional factor recognizing a specific palindromic sequence, which was originally found in the PIE of the β-casein promoter (22). STAT5 consists of two highly homologous genes defined as STAT5A and STAT5B (22) that undergo activation in response to different stimuli and exert transcriptional activation on a number of genes mainly involved in the control of cell proliferation (11). We previously demonstrated that in ECs, dm-LDL was able to induce p21waf expression by activating STAT5 (13). Herein, we demonstrate that, as a response to dm-LDL, STAT5 also becomes activated in response to gly-LDL and, consistent with its ability to bind the PIE sequence, gly-LDL was able to induce the formation of a PIE-binding complex containing STAT5. Glycated proteins are found at low levels in normal individuals during aging; however, significantly higher levels are found in certain disease states, including diabetes. In this regard, AGE level has been shown to mainly depend on a long period of poor metabolic control and correlates with the severity of some diabetes complications (28). We herein demonstrated that glycated lipoproteins generate, via RAGE engagement, a...
cascade of biochemical events that, by inhibiting cell cycle progression, may contribute to vascular complications.

AGEs exert their effects by binding to cell surface receptors and other AGE-binding proteins, including scavenger receptor type I and type II, RAGE, oligosaccharidetransferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2), and galectin-3 (AGE-R3) (29). However, both in diabetes as well as in other pathological conditions, only RAGE, among the other AGE-binding proteins, has actually been shown to transduce signals initiated by AGE ligation (6). The proximity of cells expressing RAGE to lesional areas rich in AGE and the ensuing cellular activation suggested the possibility that AGE-RAGE interaction might trigger intracellular signal transduction mechanisms altering cellular properties. Indeed, AGE ligation of RAGE activates p21\textsuperscript{ras}, recruiting downstream targets such as c-Jun NH\textsubscript{2}-terminal kinases as well as cdc2-rac GTPase (6,24,34,35). We herein provide evidence that, upon dm-LDL or gly-LDL stimulation, STAT5 becomes activated. Because the blockade of RAGE by the anti-RAGE antiserum abrogated dm-LDL-mediated effects are the following observations: 1) the effect on p21\textsuperscript{ras} expression exerted by dm-LDL and gly-LDL was almost abrogated using a blocking anti-RAGE antiserum; 2) dm-LDL did not bind to the canonical LDL receptor (13); 3) dm-LDL, as gly-LDL, induces reactive oxygen species production (13); or 4) dm-LDL was recovered from patients in which, as consequence of a long period of poor metabolic control, an irreversible rearrangement of glycated proteins has likely occurred.

RAGE is a multiligand member of the immunoglobulin superfamily of cell surface molecules (30,31) composed of an extracellular ligand–binding domain followed by a hydrophobic transmembrane-spanning domain and by a high-charged short cytosolic domain, which is essential for RAGE-mediated biological responses upon engagement of ligand (32,33). Ligation of RAGE by AGE modulates gene expression centrally by triggering a signal transduction cascade that, beside Erk1/Erk2 kinases, involved a Sin3-associated protein c-Jun NH\textsubscript{2}-terminal kinases as well as cdc2-rac GTPase (6,24,34,35). We herein provide evidence that, upon dm-LDL or gly-LDL stimulation, STAT5 becomes activated and physically interacts with RAGE, suggesting that RAGE might interact with downstream signaling molecules. Supportive of this concept is the finding that ECs transfected with the dominant-negative form of RAGE, obtained by truncation of the intracytosolic tail, displayed a marked suppression of NF-κB activation (35). However, RAGE is devoid of intrinsic catalytic activity, and STAT5 undergoes phosphorylation upon gly-LDL stimulation. Therefore, as for cytokines receptors, a non-receptor tyrosine kinase needs to be recruited. It is known that one or more members of the JAK family can be activated by a wide range of cytokines (10). Although JAK2 has been reported to play an important role in the cytokine-induced signal transduction that activates STAT5, it is still an open question whether JAK2 is the only nonreceptor tyrosine kinase responsible for the tyrosine phosphorylation of STAT5 after cytokine stimulation. Indeed, we failed to detect JAK2 tyrosine phosphorylation in response to gly-LDL in ECs (data not shown). Consistently, the addition of the JAK2 inhibitor did not affect the ability of gly-LDL to trigger STAT5 activation, suggesting
that, at least in ECs, other members of the JAKs or alternatively different nonreceptor tyrosine kinases are involved in RAGE-mediated STAT5 activation. Several reports have recently suggested that other families of tyrosine kinases, such as v-Src and v-Abl, are involved in STAT activation. Moreover, an src-dependent STAT5 activation upon IL-3, erythropoietin, or epidermal growth factor stimulation has been reported (25,36,37). We found that upon gly-LDL stimulation, the kinase activity of src becomes activated and, unlike the JAK2 inhibitor, the src kinase phosphorylates the STAT5 that migrates to the nucleus to activate p21waf expression.

AGE-RAGE interaction triggers intracellular signal transduction mechanisms, altering properties of vascular and inflammatory effector cells, thereby contributing to the impaired reparative response in AGE-rich tissue, as occurs in diabetes (2). Collectively, data presented herein imply the existence, downstream to the receptor for AGE, of a specific src-mediated STAT5-activating machinery leading to p21waf expression (Fig. 6) and that glycation represents the qualitative change of dm-LDL that account for this effect. Although the physiological role of the receptors for AGE and the means by which they are involved in the etiology of diabetic micro- and macrovascular complications needs to be clearly defined, recent clinical studies using aminoguanidine suggest that reducing AGE accumulation could benefit subjects with type 2 diabetes (38). Thus, the possibility of effective therapeutic interventions stresses the importance of detecting AGE and of elucidating the intracellular targets of AGE in order to provide the molecular basis for the development of a targeted pharmaceutical approach.

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