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A New Hypothesis for Insulin Resistance in Hypertension Due to Receptor Cleavage

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Abstract

Background—One of the most important unresolved issues in diabetes is the mechanism for the attenuated response to insulin, i.e. insulin resistance.

Aims and methods—We hypothesize that the mechanism for the insulin resistance is due to uncontrolled protease activity in the plasma, on endothelial cells and in the tissue parenchyma. To examine this hypothesis we use of microzymographic techniques in the microcirculation, plasma zymography, and receptor labeling techniques with antibodies against an extracellular domain of the insulin receptor α .

Results—The spontaneously hypertensive rat has an enhanced proteolytic activity and significant cleavage of the receptor with attenuated glucose transport. We present evidence for insulin receptor cleavage in a high fat diet and a transgenic model of diabetes.

Conclusion—These results suggest that cleavage of the extracellular domain of the insulin receptor, a situation that interferes with the ability for insulin to bind and provide an intracellular signal for glucose transport, may be involved in insulin resistance.

Keywords

Protease activity; matrix metalloproteases; insulin resistance; receptor cleavage; leukocyte; adhesion; integrin

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Introduction

By definition, the metabolic syndrome X is associated with a multitude of organ and cell dysfunction. A large body of information is available about its clinical manifestations. But few proposals have been advanced that have put in place a conceptual framework to explain the diverse cell dysfunctions that accompany the syndrome, e.g. the insulin resistance, an elevated blood pressure, a prothrombotic or proinflammatory state. It is the goal of this report to propose a conceptual mechanism for a specific condition, the insulin resistance, as a candidate for a more general mechanism that may be responsible for the diverse dysfunction in the metabolic syndrome X and in other medical conditions.

Insulin Resistance and the Multifaceted Microvascular Deficiencies in Hypertension

Many hypertensives have telltale indicators for an inflammatory cascade [1,2]. Even though traditionally recognized for its elevated arterial blood pressure, the spontaneously hypertensive rat (SHR) also has a multitude of defects that are readily visible at the level of the microcirculation. While the SHR strain has no evidence for significant abdominal obesity, it exhibits a significant insulin resistance [3–9]. The insulin resistance is present in addition to endothelial apoptosis with loss of capillaries (capillary rarefaction) [10,11], a defect in leukocyte adhesion to the endothelium [12,13] with a chronically elevated circulating leukocyte count [14] (immune suppression), a prominent defect in the response to fluid shear stress [15–18] in addition to sleep abnormalities [19]. The interesting aspect is that the insulin resistance appears in common with and yet distinct from other cell dysfunctions.

The insulin resistance in the SHR is qualitatively similar to the insulin resistance in other diabetic models, like the (Lepr^{db}) mouse model [20] or the sand rat model [21]. These models have in common a hyperglycemic state and an attenuated response to insulin [22].

Oxygen Free Radical Formation as Organ Injury Mechanism

In the past a large body of evidence in experimental models of hypertension and patients has been obtained which suggests that the enhanced organ injury in hypertension is accompanied by enhanced oxygen free radical formation (reviewed in [2,23,24]). Multiple forms of hypertension have enhanced free radical production [25–27]. In the SHR several classes of microvessels have an increased oxygen free radical formation, which if blocked leads to a reduction of blood pressure [25,28–31]. The free radical production in microvessels [32–34] is associated with enhancement of oxidase activity and reduction of enzyme activity that scavenges free radicals (e.g. superoxide dismutase, catalase) [35–37]. Reactive oxygen species modulate diverse functions [38] and exert secondary effects on microvessels, such as inhibition of endothelium-dependent vasodilation [39–42], reduced availability of nitric oxide and consequently enhanced arterial tone and blood pressure [31,43].

While oxygen free radical production has been linked to lipid peroxidation or deoxyribonucleic acid (DNA) damage, to date no conclusive mechanism has been advanced that links oxygen free radicals to specific cell dysfunctions, such as the insulin resistance. As alternative, we therefore focused on proteolytic pathway that may be associated with a complication such as insulin resistance.

Matrix Metalloproteinases Activity in the SHR, the diabetic (db/db) Mouse, and Diet-Induced Model of Obesity

The plasma protease activity in the SHR measured with a broad acting substrate (Enkzeck cleaved by metallo-, serine, acid and sulfhydryl proteases) is significantly elevated compared to the values in its normotensive control, the Wistar Kyoto (WKY) rat, or compared to the normotensive Wistar rat strain from which the SHR was generated (Figure 1). This overall protease activity in the SHR plasma detected by the broad reacting Enkzeck substrate can be reduced by a serine protease inhibitor (nafamostat mesilate; on average 20%) or by an matrix metalloproteinase (MMP) inhibitor (ethylenediaminetetraacetic acid (EDTA), 0.05 mM; on average 29%) suggesting the presence of both MMP and serine protease activity. The elevated MMP activity is also detected on microvascular endothelium by microzymography [44] with use of fluorescently quenched substrates specific for individual MMPs (Figure 2).

MMPs are expressed in a zymogen proform on endothelium and in the tissue parenchyma and under normal physiological conditions exhibit relatively low activity. Transformation of pro-MMP into an active enzyme requires cleavage or dislocation of the blocking peptide over the catalytic site of the enzyme. Among several mechanisms, a robust way to activate pro-enzymes in-vivo is by serine proteases (e.g. trypsin) [45]. Another indication that serine protease activity in the SHR may be increased, is activation of protease-activated receptor-2 (PAR-2) [46]. Treatment with angiotensin-converting enzyme (ACE) inhibitor (a serine protease inhibitor) blocks this process [46] (see below).

The protease activity in mice, homozygous for the diabetes spontaneous mutation (*Lepr^{db}*) (on the C57BLKS background, [47]), or in the sand rat model [48] remains to be determined. Development of obesity in the db/db mouse and in diet-induced model of obesity induced mRNA levels for MMP-2, MMP-3, MMP-12, MMP-14, MMP-19, and tissue inhibitor of metalloproteinase – 1 (TIMP-1) (but not TIMP-3) in obese adipose but not lean adipose tissues. Pharmacological inhibition of the MMP activity prevents the expression of CCAAT/enhancer-binding protein β , a transcription factor that appears to play a role in the adipogenesis [49].

Measurement of Insulin Receptor Cleavage in SHR

To directly examine possible cleavage of the extracellular domain of the insulin receptor we used a primary antibody against the extracellular domain of the insulin receptor alpha (R α , $\tilde{N}$ 2 sc-710 polyclonal antibody mapping to the N-terminus; 1:100) followed by a secondary antibody conjugated to the biotin/avidin with Vector NovaRED peroxidase enzyme substrate. In one set of experiments freshly harvested and heparinized cells of animals under general anesthesia (sodium pentobarbital, 50 mg/kg, i.p.) were directly labeled without centrifugation on a blood smear. Centrifugation was avoided since it may be associated with leukocyte degranulation, enzyme release, and receptor cleavage. The blood smear on a glass slide was air dried (37°C, 2 min), fixed in formalin solution, 10%, neutral buffered (1 min), and washed in deionized water (3 $\times$). The cell images were collected using a bright field microscope (40X and 60 $\times$ objective) and digitally analyzed (NIH Image J) to determine light absorption as a measure for surface density of the extracellular domain of the insulin.

Male SHR (Harlan Sprague-Dawley) at age between 14 and 16 weeks were maintained on a normal rat chow without and with a chronic MMP inhibition (doxycycline, 5.4 mg/kg/day in drinking water for 24 weeks). These SHRs have on average a plasma glucose level of 118 mg/ml and 5.7% glycated hemoglobin (%A1C) as compared to 91 mg/ml and 4.1%A1C in the WKY rats and they have a reduced density of antibody binding sites on the extracellular

domains of leukocytes and other cell types. The SHR has on average by a 5.5 % reduction of the extracellular domain density of the insulin receptor α on freshly collected and freely circulating leukocytes (Figure 3). The cleavage of the extracellular domain of the insulin receptor is significantly attenuated by chronic treatment with a broad acting MMP inhibitor (doxycycline, 5.4 mg/kg per day, 24 weeks), a process that also normalizes blood glucose and glycated hemoglobin values [44].

Since many of the circulating leukocytes in the SHR are in an activated state [17] and have high probability to be trapped in the microcirculation (instead of circulating), we also examined the insulin receptor density of naïve donor leukocytes from a Wistar strain after incubation with fresh heparinized SHR plasma (30 min exposure). This approach permits to directly examine whether the plasma of the SHR contains proteolytic activity with the ability to destroy the receptor and with minimal time for compensatory reactions (e.g. neogenesis, internalization). A period of 30 min exposure of naïve Wistar cells to SHR plasma reduces on average the extracellular binding site density of the insulin receptor α by 12% (as compared to 4% by WKY plasma and 0% by Wistar plasma). Such receptor cleavage by SHR plasma causes on average a 60% reduction of the transmembrane transport of fluorescent glucose (non-hydrolyzable glucose analog 6-NBD-deoxyglucose) into the cell cytoplasm (as compared to 13% cleavage by WKY plasma and 0% cleavage by Wistar plasma) [44].

This analysis showed that already after a 30 minute expose to SHR plasma (at room temperature), the extracellular domain receptor density on Wistar cells is significantly reduced by ~15% compared to the receptor density of the cells in control Wistar plasma. Even the WKY exhibits on average a ~4.3% reduction of the insulin receptor density, a situation that is in line with the fact that this (low pressure) control strain is not completely normal [44]. The reduction of the extracellular domain insulin receptor density is accompanied by 61% reduction of glucose transport into the cell cytoplasm of naïve control cells of Wistar rats (detected by fluorescent analog transport [50] in SHR plasma (30 min incubation) and a 13% reduction in the WKY plasma as compared to the glucose transport of Wistar cells in Wistar plasma.

Alternative Diabetic Models with Insulin Receptor Cleavage

In order to test whether insulin receptor cleavage is detectable in other models with insulin resistance, we tested receptor cleavage on two alternative diabetes models, one that depends on calorie consumption (fat sand rat) and one with a genetic defect and spontaneous mutation of leptin receptor (*Lepr^{db}* mice).

The study on the model that suffers from diabetes due to calorie overconsumption was carried out on the fat sand rat, an animal that naturally consumes low levels of calories and upon feeding normal Western diet will develop Type II diabetes. Male fat sand rats (*Psammomys obesus*, Harlan Sprague-Dawley) [48] at age between 5 and 6 weeks were transitioned after one week from a high-fiber low-calorie diet (Custom Rodent Diet, Sand Rat, 5L09; PMI Nutrition Int'l., LLC.) to a Western diet (LabDiet 5015) for a period of time between 24 and 28 weeks, while being kept on water *ad libitum*. The animals on such Western diet develop elevated plasma glucose levels (glucose levels raise from 73 ± 9 mg/ml to 344 ± 25 mg/ml, $n = 3$ rats, $P < 0.05$) and a glycated hemoglobin level (HbA1C raise from $6.2 \pm 0.2\%$ to $> 13\%$) One group of such diabetic rats was treated with an MMP inhibitor (doxycycline, 5.4 mg/kg/day in the drinking water) for a period of 24 weeks while the non-treated control group was kept on water. Treatment with the MMP inhibitor (doxycycline) served to raise the extracellular domain density of the insulin receptor by about 15% (Figure 4) in line with a significant reduction of the blood glucose (before treatment 94 ± 41 mg/ml

versus 87 ± 7 mg/ml after treatment, $n = 3$ rats) and glycated hemoglobin level (Hb A1C before treatment $7.2 \pm 1.6\%$, after treatment $6.3 \pm 0.6\%$). The evidence supports the hypothesis that an environmental risk factor by calorie overconsumption in this model causes insulin receptor cleavage.

The study on the alternative genetic model of diabetes was with a leptin receptor deficiency. Heterozygote control mice (m Lepr^{db}) (Background Strain: C57BLKS/J), homozygote Type 2 diabetic mice (Lepr^{db}) (Background Strain: C57BLKS/J), and Lepr^{db} null for TNF α (db^{TNF⁻}/db^{TNF⁻}) (Background Strain: C57BL/6J) were from Jackson Laboratory. The mice were maintained on a normal rodent chow diet. Male, 20–35 g nondiabetic m Lepr^{db} mice, 40–60 g diabetic Lepr^{db} mice and db^{TNF⁻}/db^{TNF⁻} mice were used in this study. The cross (db^{TNF⁻}/db^{TNF⁻}) of Lepr^{db} with TNF α knockout mice is heterozygous for Lepr^{db} and homozygous for TNF α knockout mice (TNF^{-/-}). The db^{TNF⁻}/db^{TNF⁻} mice show phenotypes of hyperglycemia and obesity, the diabetic phenotype that is consistent with the penetrance of the leptin receptor mutation [47,51]. The mice were examined at an age between 14 and 16 weeks. The type 2 diabetic Lepr^{db} mice had on average about a 19% reduction of the receptor labeling density, which was reduced to a non-significant 9% reduction in db^{TNF⁻}/db^{TNF⁻} (Figure 5). This evidence supports the hypothesis that genetic defects associated with insulin resistance may involve cleavage of the extracellular domain of the insulin receptor.

Thus, the underlying mechanism for the lack of insulin response in these two models and in the spontaneously hypertensive rat may be an uncontrolled degrading protease activity in the circulation and in the tissue parenchyma. Three widely different model of Type II diabetes have evidence for insulin receptor cleavage. The evidence is in line with the fact that MMP-1 (collagenase-1), MMP-2, and -9 (gelatinase A and B), MMP-7 (matrilysin), and MMP-14 (MT1-MMP) may cleave the insulin receptor alpha subunit at several positions in the extracellular domain (aa 27 to 760).

Receptor Cleavage as Cell Injury Mechanisms

Many membrane receptors have cleavage sites for MMPs. Is it possible that other membrane receptors are subject to enzymatic cleavage of their extracellular domain? The SHR suffers from a defective adhesion mechanisms of its leukocytes to the postcapillary endothelium due to a reduced CD18 integrin expression [12,13] accompanied by a chronically elevated circulating leukocyte count [14]. The extracellular domain density of CD18 on SHR leukocytes in the circulation is also reduced by SHR plasma to a similar degree as the insulin receptor. This CD18 receptor cleavage is abolished by chronic blockade of the MMP activity in the plasma, a process that also serves to return the circulating leukocyte count to normal values, likely since the circulating cell regain the ability to adhere to the vascular endothelium [44].

In a similar fashion the unchecked protease activity in the SHR leads to cleavage of the extracellular domain of the vascular endothelial growth factor receptor 2 (VEGFR2) [52]. Such receptor destruction causes apoptosis of the endothelial cells. In the case of a capillary, which are composed of single narrow endothelial tube, endothelial cell apoptosis leads to loss of the entire capillary, i.e. to capillary rarefaction, a widespread phenomenon in the SHR. Thus the combined evidence suggests the first time that cleavage of membrane receptors may constitute a more general pathophysiological mechanism.

Angiotensin-converting enzyme (ACE) Inhibitors and Matrix Metalloproteinases Blockade

A common treatment for hypertension and diabetes is with ACE inhibitors. Could they possibly interfere with receptor cleavage? ACE inhibitors block MMPs in hypertensives [53,54]. Blockade of the MMP activity in SHR with ACE inhibitors lowers blood pressure [55–57] and glucose levels in diabetes. ACE inhibitors are effective against a variety of complications in the SHR [54,58]. Disruption of the blood brain barrier in the SHR involves MMPs [59,60]. In the brain of stroke prone SHR, the main forms of MMPs involved in the extracellular matrix alterations and basal lamina changes in cerebral microvessels are MMP-2 and MMP-9 whose activity is also significantly reduced by ACE inhibitors [54,61]. Early ACE inhibition affords a long lasting inhibition of cell death in the stroke prone SHR [62]. In vitro studies have shown that ACE inhibitors are able to decrease the MMP activity directly, e.g. MMP-2 and MMP-9 are inhibited [63]. ACE inhibitors may decrease MMP activity [64] besides lowering blood pressure. Most important, many of the complications that are associated with hypertension (e.g. atherosclerosis, cerebral and cardiac lesion formation and failure) are accompanied by enhanced MMP activity and reduced TIMP activity [63,65–67]. Thus, it is apparent that ACE inhibitors have a much broader enzyme blocking profile than just preventing the formation of angiotensin II. Their full range of activities will require a broader in-vivo analysis with inclusion of other reactions involving serine proteases, MMPs, and others.

The current evidence also suggests that other protease inhibitors, such as doxycycline, may serve to reduce the protease activity in diabetes and metabolic syndrome X. Their use in diabetes remains to be determined in controlled clinical trials. We noted that the doxycycline treatment needs to be prolonged for several weeks before a reduction of protease activity in association with blood pressure reduction and blood glucose levels could be observed.

Soluble Surface Receptors

There is an increasing body of evidence to suggest that diabetics have *soluble fractions of receptors* in their plasma. Soluble insulin receptor fragments include predominantly the extracellular alpha domain of the receptor, they are present in relatively low concentrations, elevated in both Type I and Type II diabetics, and correlate with the blood glucose levels [68]. While in the past the significance of such receptor fragments in the plasma has been uncertain, their presence is in line with the current hypothesis that a proteolytic cleavage process generates them. These receptor fragments are an indicator of unchecked protease activity in the plasma and on endothelial cells of diabetics. The auto-digestion hypothesis is further supported by the evidence that soluble forms of receptors other than just the insulin receptor, e.g. the VEGFR2, are present in plasma of patients with metabolic syndrome and correlate with impaired angiogenesis [69]. The concentrations of soluble VEGFR2 were found to correlate with those of the insulin receptor, indicating that the cleavage may not be limited to single receptor types [69]. Proteolytic cleavage of surface receptor is observed under several pathophysiological circumstances [70–73] and includes soluble leukocyte adhesion receptors involved in inflammation [74].

Expert Commentary

The diversity of cell dysfunctions that accompany conditions, such as hypertension and the metabolic syndrome X, has escaped a rational explanation. The current report puts forward supporting evidence for a pathogenic mechanism that may put these mechanisms the first time under one conceptual roof that involves proteolytic degradation with direct destruction of the extracellular domain of receptors responsible for specific cell functions. Since many

membrane receptors have extracellular binding domains that may be subject to destruction by degrading proteins, such as serine or cysteine proteinases, MMPs, and others, a variety of different receptors may be compromised by such a cleavage mechanism. The evidence in this report supports this notion in the case of the insulin receptor α in multiple forms of type II diabetes and in the specific case of the β_2 integrins in the SHR [44]. In other hypertensive and diabetic models and in patients the idea remains to be tested.

Five-Year View

The cell dysfunction and end organ injury that is associated with unchecked degrading enzyme activity and direct membrane receptor damage may offer an opportunity for early intervention. Understanding the source and magnitude of these proteolytic enzymes, the reason why they are not completely blocked by native enzyme blockers (e.g. TIMPs, anti- α -trypsin), and development of novel interventions designed to block this enzyme activity in the circulation and in tissue parenchyma may open new opportunities for treatment. More sensitive techniques to measure protease activity in fresh unprocessed clinical samples need to be introduced into diagnosis [75]. There is a need to identify fragments and cleavage sites generated by enzymatic destruction of membrane receptors, a process that may in part be hampered by peptide degradation in the presence of unchecked proteolytic activity in the plasma.

Key Issues

- Evidence from three different experimental model of diabetes support the hypothesis that insulin resistance in these models is due to proteolytic cleavage of the extracellular domain of the insulin receptor, thereby preventing transmembrane signaling of the receptor after insulin binding and depression of glucose transport.
- Blockade of the unchecked protease activity with enzyme blockers serves to attenuate hyperglycemia and normalize glycated protein levels.
- The unchecked protease activity in the spontaneously hypertensive rat model also causes cleavage of other membrane receptor, e.g. CD18 on leukocytes, that leads to immune suppression, and cleavage of VEGFR2 that causes endothelial apoptosis and loss of capillaries (rarefaction). The observation provides the first time a conceptual framework for the cause of the diverse cell and organ dysfunction encountered in hypertensives and diabetics, i.e. the metabolic syndrome X.
- The presence of soluble insulin receptors in plasma of patients with Type I and Type II Diabetes is compatible with the hypothesis that receptor fragment in plasma may be generated in these patients by proteolytic cleavage.
- The enhanced proteolytic activity in the plasma and on various cell surfaces of individuals with diabetes and hypertension involves several isoforms of MMP. The MMPS are potential targets for chronic blockade. Traditional protease inhibitors, e.g. ACE inhibitors, have a broad inhibitory profile that may affect many enzyme systems; the full range remains to be investigated. Therefore the mode of action of these drugs in-vivo is only partially understood.
- The utility of MMP inhibitors in prevention of enzymatic degradation of the insulin receptor and its impact on glucose transport remains to be examined in controlled clinical trials.

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* of interest

** of considerable interest

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Figure 1.
Plasma protease activity in SHR and control WKY rats as measured by zymography.
Measurements are derived from n=6 rat plasma samples in each group. *p<0.05.

Figure 2.

Microzymography display of specific MMP activity in WKY and SHR mesentery microcirculation. (Left panels) Fluorescence micrographs generated by cleavage of intramolecularly-quenched substrate specific for MMP-7. (Right bar graphs) Fluorescent intensity values over the endothelium (I_{EC}), normalized by the average intensity on the interstitial space ($I_{Interstitial}$), for arterioles (A), capillaries and venules (V). The interstitial intensity values (not shown) in the mesentery were not significantly different between the two rat strains. Substrates specific for MMP-7 show the same localization of the fluorescent intensity over the venular endothelium ($p < 0.05$), and without significant increase over the endothelium of arterioles or capillaries (results not shown). $n = 4$ animals per group, with minimum of 4 vessels per microvessel type. * $p < 0.05$ vs WKY strain.

Figure 3. Insulin Receptor Cleavage in the Spontaneously Hypertensive Rat
 (Left) Typical micrographs of immunolabel (Vector NovaRed substrate) for the extracellular domain of the insulin receptor- α on fresh leukocytes from SHR and WKY rats before (top row) and after (bottom row) chronic treatment with MMP inhibition (doxycycline). (Right) Optical density over individual leukocytes, normalized with respect to background density, for the same groups (based on analysis of 30 cells/rat for 3 rats in each group). * $p < 0.05$ versus WKY and $\dagger\dagger P < 0.05$ versus untreated control without doxycycline.

Figure 4. Attenuation of Insulin Receptor Cleavage by MMP inhibition in the Fat Sand Rat under High Caloric Diet

(Left) Typical micrographs of immunolabel (Vector NovaRed) for the extracellular domain of the insulin receptor- α on fresh leukocytes fat sand rats before (top row) and after (bottom row) chronic treatment with MMP inhibition (doxycycline). (Right) Optical density over individual leukocytes, normalized with respect to background density, for the same groups (30 cells/rat for 3 rats in each group). * $p < 0.05$ versus WKY and $\dagger\dagger P < 0.05$ versus untreated control without doxycycline.

Figure 5. Insulin Receptor Cleavage in the Diabetic Mouse

A. Typical micrographs of immunolabel (Vector NovaRed) for the extracellular domain of the insulin receptor- α on fresh leukocytes from control mice (m Lepr^{db}), diabetic mice (Lepr^{db}) and diabetic mice null for TNF α (db^{TNF^{-/-}}/db^{TNF^{-/-}}).

B. Insulin receptor α density on leukocytes measured by light absorption after labeling with a primary antibody against the extracellular domain of the receptor and Vector NovaRed substrate. Data were normalized to m Lepr^{db} and represent mean $\pm$ standard deviation for each group. n=9 m Lepr^{db}, 11 Lepr^{db}, and 5 db^{TNF^{-/-}}/db^{TNF^{-/-}}. 152 cells from 9 m Lepr^{db}, 112 cells from 11 Lepr^{db} and 201 cells from 5 db^{TNF^{-/-}}/db^{TNF^{-/-}} were analyzed. *p< 0.05 versus m Lepr^{db} by one-way ANOVA and intergroup differences tested with Tukey