Insulin Induces Upregulation of Vascular AT₁ Receptor Gene Expression by Posttranscriptional Mechanisms

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Background—An interaction of insulin with angiotensin II effects could be pathophysiologically important for the pathogenesis of atherosclerosis and hypertension.

Methods and Results—We examined the effect of insulin on AT₁ receptor gene expression in cultured vascular smooth muscle cells (VSMCs). A 24-hour incubation with insulin (100 nmol/L) produced a 2-fold increase in AT₁ receptor density on VSMCs, as assessed by radioligand binding assays. This enhanced AT₁ receptor expression was caused by a time- and concentration-dependent upregulation of the AT₁ receptor mRNA levels, as assessed by Northern analysis. The maximal effect was detected after a 24-hour incubation of cells with 100 nmol/L insulin (270±20%). AT₁ receptor upregulation was caused by a stabilization of the AT₁ receptor mRNA, because the AT₁ receptor mRNA half-life was prolonged from 5 hours under basal conditions to 10 hours after insulin stimulation. In contrast, insulin had no influence on AT₁ receptor gene transcription, as assessed by nuclear run-on assays. The insulin-induced AT₁ receptor upregulation was followed by an increased functional response, because angiotensin II evoked a significantly elevated intracellular release of calcium in cells that were preincubated with 100 nmol/L insulin for 24 hours. The insulin-induced AT₁ receptor upregulation was dependent on tyrosine kinases, as assessed by experiments with the tyrosine kinase inhibitor genistein. Furthermore, experiments using the intracellular calcium chelator bis(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester suggest that intracellular calcium release may be involved in AT₁ receptor regulation.

Conclusions—Insulin-induced upregulation of the AT₁ receptor by posttranscriptional mechanisms may explain the association of hyperinsulinemia with hypertension and arteriosclerosis, because activation of the AT₁ receptor plays a key role in the regulation of blood pressure and fluid homeostasis. (Circulation. 1998;98:2453-2460.)

Key Words: angiotensin ■ hypertension ■ metabolism ■ genes ■ cells ■ insulin

There is evidence that hyperinsulinemia is present in diseases other than non–insulin-dependent diabetes mellitus. Among others, essential hypertension and arteriosclerotic heart disease are associated with disorders in the insulin homeostasis.1 It is well established that hypertension is closely correlated with obesity and non–insulin-dependent diabetes mellitus, and therefore it has been suggested that hyperinsulinemia and insulin resistance participate in the pathogenesis of elevated blood pressure.2 This observation is based primarily on epidemiological studies and statistical correlations, but the molecular events involved in the phenomenon are currently unknown. In this context, it has been reported that hyperinsulinemia is associated with reduced renal sodium excretion and stimulation of the sympathetic nervous system.3 Insulin is known to cause an increase of intracellular calcium and sodium in vascular smooth muscle cells (VSMCs), leading putatively to an enhanced vascular tone. Finally, insulin induces growth-promoting effects.4 However, the mechanisms involved in the correlation of hypertension and hyperinsulinemia are still poorly understood.

The renin-angiotensin system plays an important role in the control of cardiovascular function, and in addition, abnormalities in the renin-angiotensin system have been implicated in diseases such as hypertension, heart failure, and arteriosclerosis.5 The angiotensin II (Ang II) type 1 (AT₁) receptor is a G protein–coupled receptor expressed in various tissues that mediates most of the known biological effects of Ang II (eg, vasoconstriction, water retention, and vascular and cardiac hypertrophy).6 Because the AT₁ receptor expression is highly variable, its expression level may decisively influence the activity of the entire renin-angiotensin system. Indeed, it is well known that the AT₁ receptor is regulated in vivo as well as in vitro. Conditions of increased renin-angiotensin system activity cause downregulation of AT₁ receptors,7,8 whereas a decrease in the activity of the renin-angiotensin system upregulates the AT₁ receptor.9,10 Recently, it has been shown that various growth factors as well as Ang II induce a
profound downregulation of AT<sub>1</sub> receptor gene expression in cultured VSMCs. With these data in mind, we reasoned that the regulation of the AT<sub>1</sub> receptor may also be involved in the mechanisms underlying the insulin-induced elevation of blood pressure. Because smooth muscle cells are the principal target for increments of Ang II and these cells execute the AT<sub>1</sub> receptor–mediated vasoconstriction and vascular hypertrophy, we investigated the effect of insulin on AT<sub>1</sub> receptor expression in cultured VSMCs and tried to gain an insight into mechanisms participating in these regenerative pathways.

### Methods

#### Materials

Angiotensin peptides, salts, 5,6-dichlorobenzimidazole (DRB), insulin, and other chemicals were purchased from Sigma Chemical Co. [32P]dCTP, Hybond N–nylon membranes, and [125I]-labeled Ang II were obtained from Amersham. Antibiotics, serum, and cell culture medium were purchased from Gibco BRL. RNA-Clean was purchased from AGS. Oligonucleotides were synthesized with Pharmacia Chemicals, with an automated DNA synthesizer (Pharmacia LKB, gene assembler plus). Fura 2-AM was obtained from Calbiochem. Bis(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (MAPTAM), pertussis toxin (PTX), salmon sperm DNA (Sigma), and [32P]dCTP-labeled rat AT<sub>1</sub> receptor cDNA probe in the same buffer were prehybridized for 2 hours at 42°C in a buffer containing 50% formaldehyde, 0.2% SSC, 10 mmol/L denatured salmon sperm DNA (Sigma), and 10% FCS. Experiments were performed with cell cultures from passages 5 to 15.

#### mRNA Isolation and Northern Analysis

After the indicated treatments, culture medium was aspirated and the cells were lysed with 1 mL RNA-Clean (AGS), scraped, and processed according to the manufacturer’s protocol to obtain total cellular RNA. Aliquots (10 μg) were electrophoresed through 1.2% agarose 0.67% formaldehyde and stained with ethidium bromide to verify the quantity and quality of the RNA. After capillary transfer onto Hybond N membranes in 20×SSC/0.1% SDS, the RNA was cross-linked to the membranes with a Stratalinker 1800 (Stratagene). Northern blots were hybridized for 2 hours at 42°C in a buffer containing 50% deionized formamide, 0.5% SDS, 6×SSC, 10 μg/mL denatured salmon sperm DNA (Sigma), and 5×Denhardt’s solution and then were hybridized for 15 hours at 42°C with a random-primed, [32P]dCTP-labeled rat AT<sub>1</sub> receptor cDNA probe in the same buffer but without Denhardt’s solution. The rat AT<sub>1</sub> receptor cDNA probe was an 824-bp fragment generated from an AT<sub>1</sub> receptor cDNA template by the polymerase chain reaction using the primer pair 5′-GTCATGATCCCTACCCTCTACAGC-3′ and 5′-CTCGAGAACAGGAGGTTCAGGGC-3′ and Taq polymerase.

#### Radioligand Binding Assays

Experimental cells were washed 3 times with PBS. Cells were collected, and after a brief centrifugation, the pellet was resuspended in 1 mL ice-cold 50 mmol/L Tris-HCl, pH 8.0, and homogenized by repeated trituration through a 22-gauge needle. The membranes were pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. Homogenization and centrifugation were repeated twice. The final pellet was resuspended in an incubation buffer in the absence of DTTR (400 mmol/L Tris-HCl, 50 mmol/L NaH<sub>2</sub>P<sub>04</sub>, 10 mmol/L MgCl<sub>2</sub>, 0.2% BSA). Ang II receptor density and affinity were investigated in saturation experiments using increasing amounts of [125I]-labeled Ang II as radiolabeled ligand (0.125 to 2 mmol/L). Dup753 (10 μmol/L) was used to determine nonspecific binding. The assay was performed in a total volume of 250 μL incubation buffer. The incubation was carried out at 24°C for 60 minutes. These conditions allowed a complete equilibration of the receptor with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters; the filters were washed immediately 3 times with 5 mL of ice-cold incubation buffer, and radioactivity was determined in a gamma counter. All experiments were performed in triplicate. The maximal density (B<sub>max</sub>) and apparent affinity (K<sub>d</sub>) of binding sites were obtained by nonlinear regression analysis.

#### Measurement of Free [Ca<sup>2+</sup>],

VSMCs were cultured on round glass microscope slides (diameter, 12 mm) and at confluence were incubated with either vehicle or 100 mmol/L insulin for 24 hours. Subsequently, cells were washed with PBS and incubated with 2 μmol/L fura 2-AM at 37°C for 30 minutes in (in mmol/L) HEPES 20, glucose 16, NaCl 130, MgSO<sub>4</sub> 1, and CaCl<sub>2</sub> 0.5. Before the measurements, cells were rinsed gently with the same buffer containing 1 instead of 0.5 mmol/L CaCl<sub>2</sub>. The glass slides were positioned diagonally in the cuvette, and the [Ca<sup>2+</sup>]<sup>2+</sup> was measured in a Hitachi fluorescence spectrofluorometer at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm. Maximum (R<sub>max</sub>) and minimum (R<sub>min</sub>) fluorescence were determined by addition of digitonin at a final concentration of 30 mmol/L followed by the addition of Tris-base/EGTA (final concentration, 0.1 mmol/L/25 mmol/L). Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated according to Grynkiewicz et al.20

#### Nuclear Run-On Assays

These assays are a slight modification of recently described protocols. After a 24-hour incubation of VSMCs with 1 μg/mL insulin or vehicle, cells were dispersed with trypsin and washed with 150 mmol/L potassium chloride, 4 mmol/L magnesium acetate, and 10 mmol/L Tris-HCl, pH 7.4. After centrifugation in a Beckman GS-GR tabletop centrifuge with a GH 3.8 rotor (1200 rpm, 5 minutes, 4°C), the cell pellet was resuspended in 2 mL of the same buffer containing 1% Nonidet P-40 (Sigma). After lysis for 10 minutes on ice, the nuclei were isolated by centrifugation (2000 rpm, 5 minutes, 4°C) through 4 mL of 0.6 mol/L sucrose with the same equipment. The supernatants were carefully removed, and the nuclear pellet was resuspended in a buffer containing 40% glycerol, 50 mmol/L Tris, 5 mmol/L MgCl<sub>2</sub>, and 0.1 mmol/L EDTA. These were stored at −80°C until used for assays. Nuclei (~5×10<sup>10</sup> to 20×10<sup>10</sup> nuclei per reaction) were used to carry out the transcription in a reaction mixture containing 40% glycerol; 50 mmol/L Tris; 5 mmol/L MgCl<sub>2</sub>; 0.1 mmol/L EDTA; 0.5 mmol/L levels of CTP, GTP, and ATP; and 0.2 to 0.3 μmol/L [<sup>32</sup>P]UTP (>300 μCi/mmol) at 30°C for 30 minutes. Reactions were terminated by addition of 800 μL of RNA-Clean, and the radioactive RNA was isolated and purified by collection of the eluate from a Bio-Rad P-30 spin column. [<sup>32</sup>P]UTP-labeled RNA (~5×10<sup>10</sup> to 1×10<sup>10</sup> cpm) was dissolved in hybridization solution (100 mmol/L TES, pH 7.4, 0.3 mol/L NaCl, and 100 μg/mL Escherichia coli tRNA). Plasmids (5 μg) containing cDNAs for the AT<sub>1</sub> receptor (a HindIII-NcoI cDNA insert from pc71a1b subcloned in pK5s+ Bluescript) or GAPDH (rat GAPDH in pBlI30; International Biotechnology) and a plasmid (KS+—Bluescript) without insert were linearized, denatured, and applied to nylon membranes by use of a dot-blot apparatus. These membranes were prehybridized for 2 hours at 42°C in 100 mmol/L TES, 0.3 mol/L NaCl, 100 μg/mL E. coli tRNA, and 5×Denhardt's solution and were hybridized at 42°C for 16 hours. Membranes were washed for 10 minutes at room temperature in 2×SSC and for 15 minutes at 50°C in 2×SSC/0.1% SDS. The filters were exposed to film for 12 to 48 hours, and autoradiographic signals were quantified by laser densitometry.
Statistical Analysis
Data are presented as mean±SEM. Statistical analysis was performed with the ANOVA test.

Results
Cells were grown to confluence, and serum was removed from the culture medium 24 hours before initiation of experimental treatments to obviate its effects.

Figure 1 illustrates autoradiographic results from Northern hybridization of a rat vascular AT1 receptor cDNA probe to Northern blots of 10 μg total RNA extracted from VSMCs at indicated time points. Hybridization of a GAPDH cDNA probe to same blot stripped of AT1 receptor cDNA probe. Representative of 5 separate experiments.

Under basal conditions, the AT1 receptor mRNA remained unchanged over the time course of the experiment. Furthermore, the autoradiogram revealed a time-dependent elevation of the transcript level. The AT1 receptor mRNA signal appeared to be significantly increased 12 hours after exposure to insulin, and this increase was sustained for up to 24 hours. Also shown in Figure 1 is hybridization of a GAPDH cDNA probe to the same Northern blot. GAPDH mRNA appeared to be stable over the time course of the
AT1 Receptor Density in Vehicle and Insulin-treated Cells

Figure 3. Effect of insulin on membrane receptors by saturation binding with [125I]-labeled Ang II. Confluent cells on 24-well culture plates were exposed to either vehicle or 100 nmol/L insulin. Saturation binding assays using [125I]-labeled Ang II were performed on isolated cell membranes. AT1 receptor antagonist Dup753 (10 μmol/L) was used to define nonspecific binding. Each curve represents specific binding of radioligand (cpm radioligand bound minus cpm bound in presence of 10 μmol/L Dup753). Kd and Bmax values reported in text were derived from nonlinear regression of specific bound vs free data. Each point represents binding data of 3 independent experiments ±SEM.

De Novo mRNA Synthesis in VSMC

Figure 4. Nuclear run-on assays. Cells were challenged with either vehicle or 100 nmol/L insulin for 24 hours, and nuclei were isolated and used for nuclear run-on assays. Radioactive de novo-synthesized mRNA was hybridized to AT1 receptor cDNA (AT1-R), GAPDH cDNA (GAPDH), and KS1-Bluescript plasmid (KS1 BS) in which AT1 receptor cDNA had been cloned. Top, Representative autoradiogram; bottom, densitometric analysis of 3 separate experiments (mean ± SEM).
regulation of AT₁ receptor mRNA hybridization signal relative to vehicle-treated control levels at 0 hours. A 12-hour incubation with 100 nmol/L insulin caused an upregulation of AT₁ receptor mRNA levels to 231±33%. After 24 hours of insulin stimulation, the AT₁ receptor mRNA signals were measured at 270±20% relative to the control level at 0 hours (100%). GAPDH mRNA expression was not significantly regulated by insulin. Figure 2, bottom, demonstrates the concentration-dependent effect of insulin on AT₁ receptor mRNA expression. Cells were challenged with vehicle or 0.01 to 100 nmol/L insulin. AT₁ receptor upregulation was significant with 0.1 nmol/L insulin (153±12.7% of control) and reached a maximum at 100 nmol/L insulin. These data demonstrate that insulin specifically induces the upregulation of AT₁ receptor mRNA in VSMCs.

To assess whether the increased level of AT₁ receptor mRNA was translated to an elevation of AT₁ receptor protein expression, radioligand binding assays were performed. Therefore, AT₁ receptor binding sites were measured in a membrane-binding assay after a 24-hour treatment of VSMCs with either 100 nmol/L insulin or vehicle. Nonlinear regression analysis demonstrated that [¹²⁵I]-labeled Ang II bound to a single population of sites, as expected for VSMCs (data not shown). Figure 3 shows graphically the [¹²⁵I]-labeled Ang II saturation binding to VSMCs treated with either insulin or vehicle. Binding to vehicle-treated cells revealed a $K_d$ value of 0.33 (0.2 to 0.47) fmol/L and a $B_{max}$ value of 389±18.6 fmol/mg protein. Binding to insulin-treated cells showed an increase in the $B_{max}$ value to 776±33.5 fmol/mg protein, without changes in the affinity for the radioligand ($K_d$ value, 0.26 [0.15 to 0.36] fmol/L). These binding data indicate that insulin markedly elevates AT₁ receptor protein expression as a result of an enhanced level of AT₁ receptor mRNA.

Experiments were performed to gain insight into general mechanisms participating in the insulin-induced elevation of AT₁ receptor expression. The insulin-induced upregulation of the AT₁ receptor mRNA that causes an elevation of AT₁ receptor density could mechanistically be based either on an enhanced gene transcription rate or on posttranscriptional modulations of the AT₁ receptor mRNA.

To measure the de novo synthesis of the AT₁ receptor mRNA, nuclei from VSMCs incubated for 24 hours with either 100 nmol/L insulin or vehicle were isolated and used in a nuclear run-on assay. Figure 4 illustrates a representative autoradiogram of radiolabeled de novo synthesized mRNA to AT₁ receptor, GAPDH, and plasmid DNA. Also shown is the densitometric analysis of 3 separate experiments. Incubation with insulin, which had caused significant upregulation of AT₁ receptor mRNA, had no effect on AT₁ receptor transcription rate. These experiments suggested that the detected enhancement of AT₁ receptor mRNA and protein by insulin was not mediated through an increase in de novo AT₁ receptor gene synthesis.

Consequently, we assumed that the insulin-induced elevation in AT₁ receptor gene expression may be caused by posttranscriptional mechanisms. Therefore, after a 24-hour treatment with either vehicle or 100 nmol/L insulin, gene transcription of VSMCs was inhibited by incubation with 50 μg/mL of the RNA–polymerase II inhibitor DRB. Northern hybridizations were then performed on RNA extracted from VSMCs at the indicated time points. In vehicle-treated cells, AT₁ receptor mRNA levels were reduced to 50% of control levels ~5 hours after the addition of DRB (Figure 5). This rate of AT₁ receptor mRNA decay was taken to represent a measure of mRNA stability under otherwise normal conditions. This decay rate contrasted markedly with the degradation for AT₁ receptor mRNA in cells pretreated with insulin: preincubation of VSMCs with insulin produced a marked increase in AT₁ receptor mRNA stability with an AT₁ receptor mRNA half-life calculated at ~10 hours. These data suggest that insulin-induced enhancement of AT₁ receptor mRNA stability rather than modulation of AT₁ receptor transcription rate is involved in the insulin-induced upregulation of AT₁ receptor gene expression.

Insulin receptor activation by its ligand causes stimulation of various intracellular pathways. To investigate whether involvement of some of these intracellular signals is a prerequisite for insulin-induced AT₁ receptor upregulation, cells were preincubated with various agents that selectively block particular signaling pathways of insulin. Incubation with the tyrosine kinase inhibitor genistein...
Intracellular pathways involved in insulin-induced AT1 receptor regulation

(1 μmol/L) for 30 minutes before either 100 nmol/L insulin or vehicle was added to the culture medium effectively inhibited the insulin-induced AT1 receptor upregulation, suggesting that tyrosine phosphorylation is essentially required for this receptor regulation (Figure 6A). Pretreatment with PTX did not influence insulin-induced AT1 receptor regulation (Figure 6B), suggesting that coupling of insulin receptors to G proteins is not involved in the observed effects. The intracellular calcium chelator MAPTAM caused a moderate AT1 receptor downregulation. The insulin-induced AT1 receptor upregulation was prevented by MAPTAM (Figure 6C). 18S rRNA expression and viability of VSMCs was monitored throughout the experiments with genistein, PTX, and MAPTAM. There were no significant changes in cell appearance or 18S rRNA expression in this experimental setup (data not shown).

We further reasoned that upregulation of AT1 receptor gene expression should consequently lead to an enhanced functional response of VSMCs on Ang II stimulation. To test this, we examined Ang II–induced elevation of [Ca^{2+}]. Figure 7 illustrates a representative time course of [Ca^{2+}], of VSMCs pretreated for 24 hours with either 100 nmol/L insulin or vehicle. Basal [Ca^{2+}], was measured at ≈30 nmol/L. After 1 minute, the cells were challenged with 100 nmol/L Ang II. Calculation of 4 separate experiments revealed that 100 nmol/L Ang II induced a maximal [Ca^{2+}], increase of 172.5±20.5 nmol/L in vehicle-treated VSMCs, whereas the same concentration of Ang II caused a maximal [Ca^{2+}], increase of 292.5±37 nmol/L in insulin-pretreated VSMCs. The Ang II–induced elevation of [Ca^{2+}], was blocked by addition of an AT1 receptor antagonist (Dup753) but not by an AT2 receptor antagonist (PD123177) (data not shown). These data demonstrate that the insulin-induced upregulation
Angiotensin II-induced [Ca^{2+}]i in VSMC

**Vehicle-pretreated VSMC**

**Insulin-pretreated VSMC**

**Figure 7.** Time course of Ang II–induced elevation of [Ca^{2+}]i. Cells were seeded on round glass slides, grown to confluence, and preincubated with either vehicle (top) or 100 nmol/L insulin (bottom) for 24 hours. After pretreatment of cells as in Experimental Procedures, VSMCs were challenged with 100 nmol/L Ang II, and [Ca^{2+}]i was measured. Data represent 4 separate experiments.

AT1 receptor gene expression. In contrast, LDL leads to an upregulation of the AT1 receptor in vitro as well as in vivo stabilization of AT1 receptor mRNA in cultured VSMCs. The AT1 receptor–mediated Ang II stimulation. This observation associated the AT1 receptor and causes an enhanced biological response to Ang II with regard to elevated blood pressure but did not establish the molecular events that may have participated in this phenomenon. Because only correlations have been described, it has not been clarified whether insulin itself could cause the observed effects. Our data suggest that insulin induces an upregulation of the AT1 receptor, and therefore the concept of hyperinsulinemia-induced hypertension via upregulation of AT1 receptor gene expression seems to be very reasonable, especially because insulin-induced attenuation of sodium excretion and activation of the sympathetic system synergistically with AT1 receptor overexpression, could affect pathophysiological blood pressure regulation.

On the basis of our data, the regulation of the AT1 receptor takes place at the posttranscriptional level. Insulin leads to AT1 receptor upregulation more likely via stabilization of the AT1 receptor mRNA than via activation of AT1 receptor gene transcription. Indeed, the posttranscriptional regulation seems to be of greater relevance than the transcriptionally induced AT1 receptor regulation in the case of Ang II–, growth factor–, or LDL-induced AT1 receptor regulation as well.11–13,23 AT1 receptor mRNA binding proteins, which bind at the 3′-untranslated region of the AT1 receptor mRNA, are potentially involved in the inducible (de)stabilization of the AT1 receptor mRNA. At present, the intracellular pathways that connect activation of a receptor at the cell surface with this machinery of posttranscriptional regulation are only poorly understood. Insulin receptor activation by its ligand causes stimulation of multiple intracellular pathways, including various phosphorylation processes, G-protein coupling, calcium signaling, phosphatidylinositol-3-kinase and MAP kinase activation, and interference with the cAMP turnover.29 According to our findings, AT1 receptor mRNA is stabilized by insulin through tyrosine phosphorylation and MAP kinase–dependent intracellular pathways. It is attractive to speculate that MAP kinase signaling is involved in the induction of mRNA binding proteins, which then influence AT1 receptor mRNA turnover. This is in good agreement with previous findings that associated MAP kinase activation with postranscriptional mRNA processing of various genes.29 Intracellular calcium also participates in the regulatory processes of AT1 receptor expression, because MAPTAM slightly downregulates the AT1 receptor mRNA. Nevertheless, it is not probable that the insulin-induced AT1 receptor upregulation is specifically blocked by MAPTAM. Moreover, MAPTAM-induced inhibition of the insulin effect on AT1 receptor expression may be due to the initial decrease of AT1 receptor mRNA baseline levels. The results presented provide novel insights...
into the mechanisms involved in AT_{1} receptor regulation and will initiate further investigations dealing with this complicated intracellular mechanism governing the modulation of gene expression.

The insulin-induced AT_{1} receptor regulation may be of fundamental importance for the treatment of patients suffering from hypertension and hyperinsulinemia, because it favors therapy with AT_{1} receptor antagonists or ACE inhibitors. These therapeutics reduce AT_{1} receptor activation and may therefore improve the outcome in these syndromes.

**Acknowledgments**

This work was supported by the Deutsche Forschungsgemeinschaft. The excellent technical assistance of Marc Wolff is gratefully appreciated.

**References**

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Circulation. 1998;98:2453-2460
doi: 10.1161/01.CIR.98.22.2453

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