Growth hormone is a positive regulator of adiponectin receptor 2 in 3T3-L1 adipocytes

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Abstract The fat-derived protein adiponectin is an important insulin-sensitizing adipocytokine which is downregulated in insulin resistance and obesity. Recently, two receptors of this adipose-expressed protein called adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) have been cloned. To clarify expression and regulation of these receptors in fat cells, AdipoR1 and AdipoR2 mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction during differentiation of 3T3-L1 adipocytes and after treatment with various hormones known to induce insulin resistance. Interestingly, AdipoR2 synthesis was significantly increased up to 4.8-fold during differentiation of 3T3-L1 preadipocytes, whereas AdipoR1 expression was only augmented up to 1.4-fold. Furthermore, growth hormone (GH) induced AdipoR2, but not AdipoR1 mRNA by up to 2.4-fold in a dose- and time-dependent fashion with significant stimulation detectable at concentrations as low as 5 ng/ml GH and as early as 2 h after effector addition. The positive effect of GH on AdipoR2 expression could be reversed by withdrawal of the hormone for 24 h. In contrast, other key hormones involved in the regulation of insulin resistance and energy metabolism such as insulin, isoproterenol, dexamethasone, triiodothyronine, angiotensin 2, tumor necrosis factor α, and interleukin-6 did not influence AdipoR1 and AdipoR2 synthesis in vitro. Taken together, our results suggest that AdipoR2 expression is differentiation-dependent and selectively regulated by GH implying a potential role of this hormone in adiponectin-associated alterations of insulin sensitivity and energy homeostasis.

Key words: 3T3-L1 adipocyte; Adiponectin; Adiponectin receptor; Diabetes; Growth hormone; Insulin resistance; Obesity

1. Introduction

Insulin resistance is frequently associated with obesity and is a major risk factor for the development of type 2 diabetes, hypertension, and coronary artery disease. In recent years, the molecular link between increased adiposity and reduced sensitivity of target tissues to insulin has become more evident [1]. Thus, it has been shown that adipocytes secrete biologically active proteins which profoundly influence glucose metabolism. Among those so-called adipocytokines, tumor necrosis factor (TNF) α [2], interleukin-6 (IL-6) [3], and resistin [4] decrease insulin sensitivity in vivo and in vitro. Recently, it has been shown that another fat-derived factor, adiponectin, is an insulin-sensitizing adipocytokine, replenishment of which increases insulin sensitivity in different models of insulin resistance in vivo [5,6]. Adiponectin was originally identified by four independent groups using different experimental approaches and is, therefore, also called Acrp30, GBP28, apM1, or AdipoQ [7–10]. The protein is a member of the soluble defense collagen superfamily, and adiponectin expression is decreased in murine and human obesity and insulin resistance [9,11,12]. Evidence that adiponectin is not simply a factor passively regulated by insulin resistance and obesity, but actively influences these states has been presented by different groups [5,6,13,14]. In accordance with adiponectin being an endogenous insulin sensitizer, various insulin resistance-inducing hormones including insulin, β-adrenergic agonists, glucocorticoids, TNFα, and IL-6 decrease expression of this adipocytokine in fat cells in vitro [15–17]. Insulin-sensitizing thiazolidinediones, on the other hand, profoundly stimulate adiponectin synthesis in vivo and in vitro [18,19]. Very recently, two receptors for adiponectin were cloned by Yamauchi et al. and were named adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) [20]. The authors demonstrate that AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly found in liver [20]. Until now, it has not been determined whether adiponectin receptors are also expressed in adipocytes and might, therefore, potentially mediate paracrine effects of adiponectin on fat cells. Furthermore, it is tempting to speculate that insulin resistance-inducing hormones might decrease insulin sensitivity at least partly by downregulating either adiponectin receptor.

In the current study, we therefore studied expression of adiponectin receptors in 3T3-L1 adipocytes and examined the effect of differentiation, and major hormones associated with the regulation of energy balance and insulin resistance such as insulin, isoproterenol, dexamethasone, triiodothyronine (T3), growth hormone (GH), angiotensin 2 (AT2), TNFα, and IL-6 on AdipoR1 and AdipoR2 synthesis in vitro. We demonstrate for the first time that AdipoR1 and AdipoR2 are expressed in 3T3-L1 adipocytes and are induced during in vitro differentiation. Furthermore, we provide first evidence that GH is a potent, selective, and reversible stimulator of AdipoR2 synthesis in 3T3-L1 fat cells.

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2. Materials and methods

2.1. Materials

AT2, dexamethasone, GH, IL-6, isobutylmethylxanthine, isoproterenol, T3, and TNFα were purchased from Sigma (St. Louis, MO, USA), insulin from Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany), cell culture reagents from Life Technologies (Grand Island, NY, USA).

2.2. Cell culture and differentiation

3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD, USA) were grown and differentiated into fat cells as previously described [21]. In brief, cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 10% fetal bovine serum (FBS), and antibiotics (culture medium) and confluent preadipocytes were induced for 3 days in culture medium further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone. Then, they were maintained for another 3 days in culture medium with 1 μM insulin and for an additional 4–8 days in culture medium. After this period, more than 90% of the cells showed fat droplet accumulation.

2.3. Analysis of AdipoR1 and AdipoR2 gene expression

AdipoR1 and AdipoR2 gene expression was measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) in a fluorescent temperature cycler (Taqman, Applied Biosystems, Darmstadt, Germany) as described recently [22]. Briefly, total RNA was isolated from 3T3-L1 cells using TRIzol (Life Technologies) and 1 μg RNA was reverse-transcribed with standard reagents (Life Technologies). 2 μl of each RT reaction was amplified in a 26 μl PCR by using the Brilliant SYBR Green QPCR Core Reagent Kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. Samples were incubated in the Taqman for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The following primers were used: AdipoR1 (accession number BC014875) CCCCCTTACCCCCGTCCTTAC (sense) and GGCGTGGCTTTGTTTGTCCTA (antisense); AdipoR2 (accession number XM_132831) TGCGCACACGTTTCAGTCTCCT (sense) and TTCTATGATCCCCAAAAGTGTGC (antisense); 36B4 (accession number NM007475) AAGCGCGTCCTGGCATTGTCT (sense) and CCGCAGGGGCAGCAGTGGT (antisense). SYBR Green I fluorescence emissions were monitored after each cycle. Expression of AdipoR1, AdipoR2, and 36B4 mRNA was quantified by the second derivative maximum method of the Taqman Software (Applied Biosystems) determining the crossing points of individual samples by an algorithm which identifies the first turning point of the fluorescence curve. AdipoR1 and AdipoR2 expression was calculated relative to 36B4 which was used as an internal control due to its resistance to hormonal regulation [23–25]. Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

2.4. Statistical analysis

Results are expressed as mean ± S.E.M. Differences between treatments were analyzed by unpaired Student's *t*-tests. P values < 0.05 are considered significant, < 0.01 highly significant.

3. Results

3.1. Quantification of AdipoR1 and AdipoR2 mRNA levels in 3T3-L1 adipocytes

First, the reliability of quantitative real-time RT-PCR was tested. For this purpose, increasing amounts of total cellular RNA from differentiated 3T3-L1 cells were reverse-transcribed and analyzed using specific primer pairs for AdipoR1 and AdipoR2 (Fig. 1A,B). Linearity between total RNA used per reaction and amount of mRNA measured by the Taqman software was obtained between 2 and 200 ng of total RNA for both mRNA products (Fig. 1A,B).

3.2. AdipoR1 and AdipoR2 mRNA expression is stimulated during differentiation

Expression of AdipoR1 and AdipoR2 mRNA during differentiation was determined. As compared to undifferentiated preadipocytes, AdipoR1 synthesis increased significantly 1.4-fold on day 9 of differentiation ($P < 0.01$) (Fig. 2A). Induction of AdipoR2 mRNA expression was more pronounced with a significant 3.7- and 4.8-fold stimulation seen on days 6 and 9 of differentiation, respectively, as compared to undifferentiated cells ($P < 0.01$) (Fig. 2B).

3.3. GH stimulates AdipoR2 gene expression in a dose- and time-dependent manner

To investigate the regulation of AdipoR1 and AdipoR2

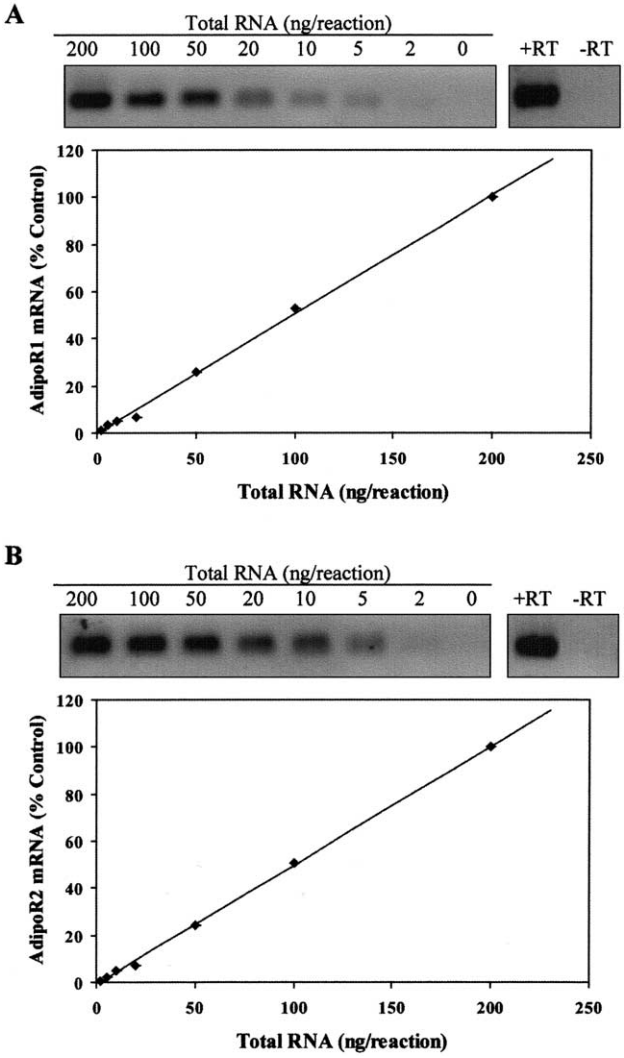

Fig. 1. Measurement of AdipoR1 and AdipoR2 mRNA levels in 3T3-L1 adipocytes. Increasing amounts of total RNA from fully differentiated 3T3-L1 cells were subjected to quantitative real-time RT-PCR with primers specific for (A) AdipoR1 and (B) AdipoR2 as described in Section 2. Data are expressed relative to mRNA levels measured with 200 ng RNA (= 100%). Top panels, agarose gel electrophoresis of the PCR products at cycle 28. Furthermore, PCR products after 40 cycles are shown in samples which were reverse-transcribed in the presence (+) or absence (−) of RT.

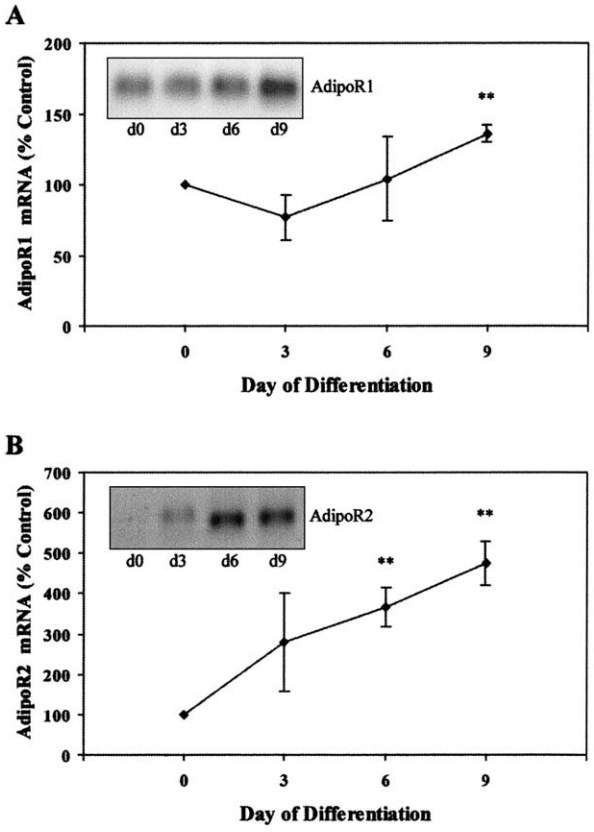

Fig. 2. Differentiation-dependent AdipoR1 and AdipoR2 gene expression. Confluent preadipocytes (day 0) were differentiated and on the indicated days (0, 3, 6, 9) total RNA was subjected to quantitative real-time RT-PCR. AdipoR1 (A) and AdipoR2 (B) mRNA levels are shown relative to cells on day 0 (= 100%). Results are the means ± S.E.M. of four independent experiments. $^{**}P < 0.01$ comparing confluent cells (day 0) with differentiated adipocytes (day 6 and 9). Insets, agarose gel electrophoresis of the PCR products for (A) AdipoR1 and (B) AdipoR2 at cycle 23.

gene expression in vitro, 3T3-L1 adipocytes were incubated for 16 h with various hormones which have been shown to cause insulin resistance and mRNA levels of both receptors were measured by quantitative RT-PCR. Treatment of 3T3-L1 cells with 100 nM insulin, 10 μM isoproterenol, 100 nM dexamethasone, 1 μM T3, 500 ng/ml GH, 10 μM AT2, or 10% FBS did not significantly influence AdipoR1 expression (Fig. 3A). Furthermore, incubation of differentiated 3T3-L1 cells with insulin (100 nM), isoproterenol (10 μM), GH (500 ng/ml), TNFα (10 ng/ml), and IL-6 (30 ng/ml) for shorter periods of time did not significantly influence AdipoR1 synthesis (Fig. 3C). A significant 2.3-fold induction of AdipoR2 expression could be observed when 3T3-L1 adipocytes were treated with 500 ng/ml GH for 16 h ($P < 0.01$) (Fig. 3B). Interestingly, addition of 10% FBS to the starving medium for 16 h significantly induced AdipoR2 expression 1.9-fold ($P < 0.01$) (Fig. 3B). In contrast, mRNA levels of AdipoR2 were not influenced by insulin, isoproterenol, dexamethasone, T3, AT2, TNFα, and IL-6 (Fig. 3B,D).

GH increased AdipoR2 expression in a dose-dependent fashion. Thus, significant 1.9-fold induction of AdipoR2 mRNA was detectable at GH concentrations as low as 5 ng/ml ($P < 0.01$), and a maximal 2.4-fold increase was found at 500 ng/ml effector ($P < 0.01$) (Fig. 4A). Furthermore, AdipoR2 gene expression was stimulated in a time-dependent manner using GH at a concentration of 500 ng/ml (Fig. 4B) or 50 ng/ml (Fig. 4C). For both conditions, significant stimulation of AdipoR2 mRNA was first seen at 2 h of GH addition with maximal effect observed after 8 h of effector and induction persisting for up to 24 h (Fig. 4B,C).

3.4. *Stimulation of AdipoR2 gene expression by GH is reversible*

Finally, we determined whether the stimulatory effect of GH on AdipoR2 gene expression was reversible. Fully differentiated 3T3-L1 adipocytes were treated with GH (500 ng/ml) for 16 h and the medium was then replaced by DMEM containing 25 mM glucose and 10% FBS for an additional 24 h. Addition of GH again increased AdipoR2 mRNA expression by about 2.4-fold as compared to untreated control cells ($P < 0.01$) (Fig. 5, columns 1 and 2). However, removal of GH from the medium for 24 h decreased AdipoR2 mRNA expression to control levels (Fig. 5, columns 3 and 4). Interestingly, addition of serum-containing medium for an additional 24 h again stimulated AdipoR2 gene expression about 1.6-fold (Fig. 5, columns 1 and 3). However, it has to be pointed out that the decrease in AdipoR2 expression in the wash-out period after GH treatment (Fig. 5, columns 2 and 4) and the increase in the wash-in period of the control experiment (Fig. 5, columns 1 and 3) did not reach statistical significance.

4. Discussion

Adiponectin is an important adipocytokine with insulin-sensitizing effects and might represent a novel treatment target for insulin resistance and type 2 diabetes [5,6]. The mechanisms by which adiponectin decreases insulin resistance have been better elucidated in recent months. Thus, a globular fragment most potently increases fatty acid combustion in muscle cells, whereas only full-length adiponectin augments insulin-induced inhibition of glucose output in liver cells in vivo and in vitro [5,6,26]. Furthermore, activation of adenosine monophosphate kinase (AMPK) followed by inhibition of acetyl coenzyme A carboxylase (ACC), as well as stimulation of peroxisome proliferator-activated receptor α, might be mechanisms for the insulin-sensitizing effects of this adipocytokine [26,27]. Very recently, AdipoR1 and AdipoR2 have been cloned as two adiponectin receptors [20]. Both proteins are predicted to contain seven transmembrane domains which are structurally and functionally distinct from G protein-coupled receptors [20]. AdipoR1 is preferentially expressed in muscle and appears as a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin, whereas AdipoR2 is abundantly found in liver and serves as an intermediate-affinity receptor for both forms of adiponectin [20]. In the current study, we show for the first time that both adiponectin receptors are also expressed in 3T3-L1 adipocytes and are induced during fat cell differentiation. These findings imply that adiponectin might alter insulin sensitivity and metabolism of adipocytes in a paracrine manner besides its well-known effects on muscle and liver cells. Consistent with this hypothesis, Wu et al. recently demonstrated activation of AMPK followed by inhibition of ACC by globular adiponectin in rat adipocytes [28]. Furthermore, globular adiponectin further enhanced insulin-stimulated glu-

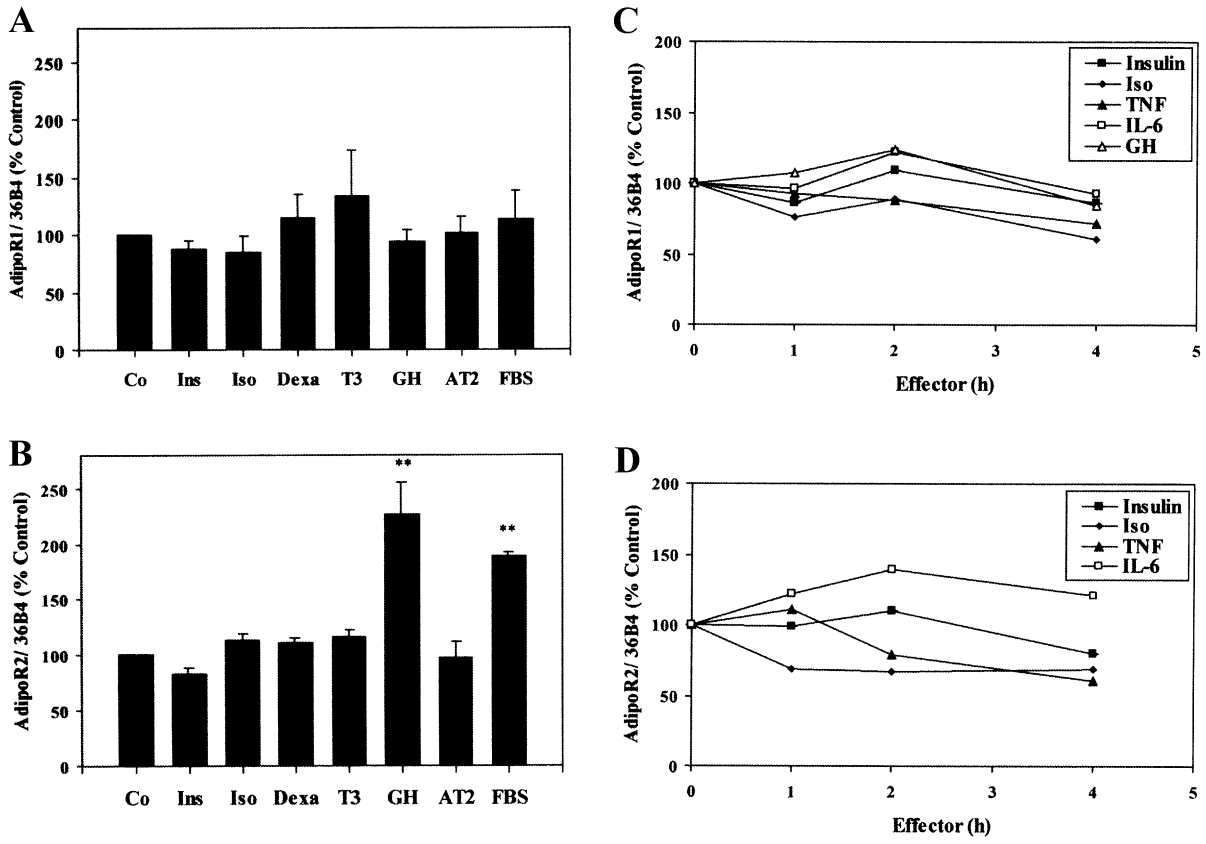

Fig. 3. Hormonal control of AdipoR1 and AdipoR2 gene expression. Fully differentiated 3T3-L1 adipocytes were (A,B) serum-deprived for 6 h before insulin (Ins, 100 nM), isoproterenol (Iso, 10 μM), dexamethasone (Dexa, 100 nM), T3 (1 μM), GH (500 ng/ml), AT2 (10 μM), and 10% FBS were added for 16 h or (C,D) serum-starved overnight before insulin (100 nM), isoproterenol (Iso, 10 μM), TNFα (TNF, 10 ng/ml), IL-6 (30 ng/ml), and GH (500 ng/ml) were added for up to 4 h. Total RNA was subjected to quantitative real-time RT-PCR to determine (A,C) AdipoR1 and (B,D) AdipoR2 mRNA levels normalized to 36B4 as described in Section 2. Gene expression is shown relative to untreated control (Co) cells (= 100%). Results are (A,B) the means ± S.E.M. of at least four and (C,D) the means of at least two independent experiments. $^{**}P < 0.01$ comparing GH- and FBS-treated with untreated cells.

cose uptake at submaximal insulin concentrations and reversed the inhibitory effect of TNFα on insulin-stimulated glucose uptake [28].

Various hormones have been shown to induce insulin resistance. However, molecules that mediate these effects are not well defined. Among those, GH, which is produced primarily in the anterior pituitary gland as a 22 kDa polypeptide, potently antagonizes insulin action on insulin-sensitive tissues such as muscle, fat, and liver in vivo and in vitro [29]. Thus, it has been shown that patients with GH excess due to pituitary tumors are insulin-resistant [30,31]. The role of GH in common insulin resistance and obesity is less clear. On the one hand, it has been suggested that nocturnal GH secretion in diabetic patients may contribute to nocturnal hyperglycemia [32]. On the other hand, in obese insulin-resistant individuals basal and stimulated GH secretion are low [33,34]. In the current study, we demonstrate for the first time that AdipoR2 but not AdipoR1 mRNA is potently induced by GH. These findings indicate that AdipoR2 might be a mediator of some effects of GH on fat cell metabolism. Thus, it appears possible that upregulation of AdipoR2 by GH sensitizes adipocytes to the effects of adiponectin on insulin sensitivity [28]. Since GH has been shown to potently induce insulin resistance by uncoupling phosphatidylinositol 3-kinase and its downstream signals in 3T3-L1 cells [35], upregulation of AdipoR2 might be a compensatory mechanism by which insulin sensitivity is at least partially restored. Furthermore, the effect of GH on AdipoR2 gene expression is reversible as removal of the hormone for 24 h decreases AdipoR2 mRNA to control levels. Moreover, AdipoR2 is significantly induced by addition of FBS which points to the fact that GH might also have a strong positive effect in vivo. However, we cannot exclude the possibility that other, nontested hormones present in FBS upregulate AdipoR2 but not AdipoR1.

Increased serum levels of insulin, catecholamines, glucocorticoids, thyroid hormones, AT2, TNFα, and IL-6 have been shown to impair glucose tolerance profoundly. On the molecular level, decreased activity of insulin signaling proteins such as insulin receptor substrate molecules which are essential for insulin action contributes to insulin resistance induced by these hormones [36–43]. Furthermore, we and others have demonstrated that insulin, β-adrenergic agonists, TNFα, and IL-6 impair insulin sensitivity at least in part by decreasing adiponectin and stimulating IL-6 expression in fat cells [15–17,44]. In our in vitro system, insulin, isoproterenol, dexamethasone, T3, AT2, TNFα, and IL-6 do not significantly affect AdipoR1 and AdipoR2 gene expression. Therefore, it is unlikely that at least in adipocytes these hormones mediate their insulin resistance-inducing effects via downregulation of either receptor.

In summary, we demonstrate for the first time that Adi-

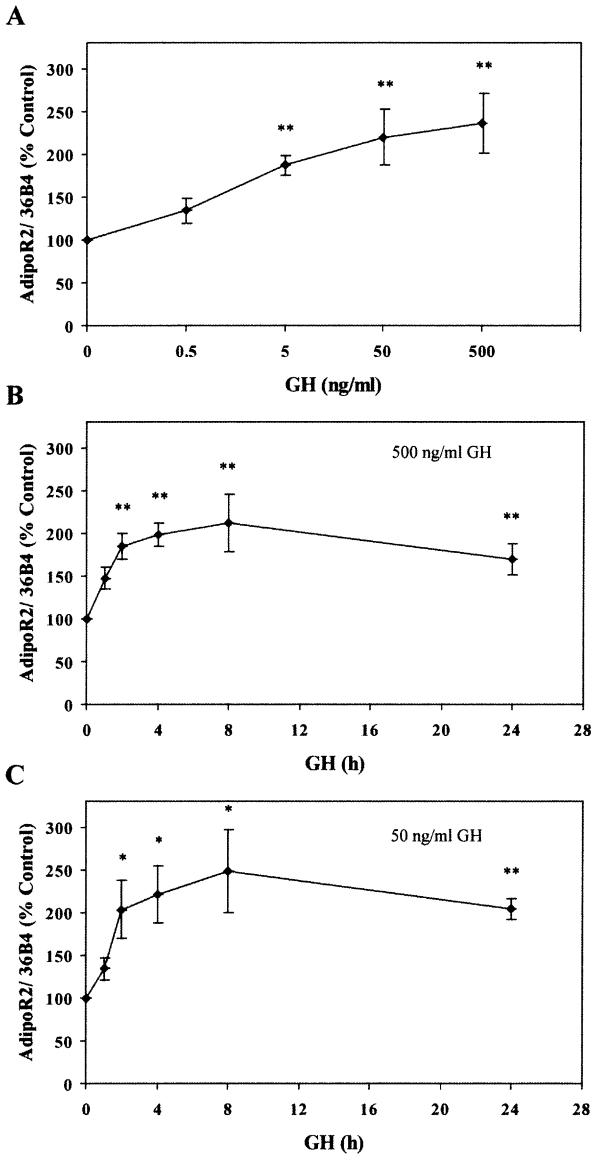

Fig. 4. Dose- and time-dependent stimulation of AdipoR2 gene expression by GH. 3T3-L1 cells were serum-starved before (A) various concentrations of GH were added for 16 h or (B) 500 ng/ml and (C) 50 ng/ml GH were added for different periods of time. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine AdipoR2 mRNA levels normalized to 36B4 expression as described in Section 2. Data are expressed relative to untreated control cells (=100%). Results are the means ± S.E.M. of at least three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ comparing GH-treated with untreated cells.

poR1 and AdipoR2 are expressed in 3T3-L1 fat cells and are upregulated during differentiation of preadipocytes. Furthermore, we show that GH is a highly selective positive regulator of AdipoR2 but not AdipoR1 synthesis. Further work is needed to elucidate the role of this regulation for GH-associated disease states and effects on other insulin-sensitive tissues such as muscle and liver.

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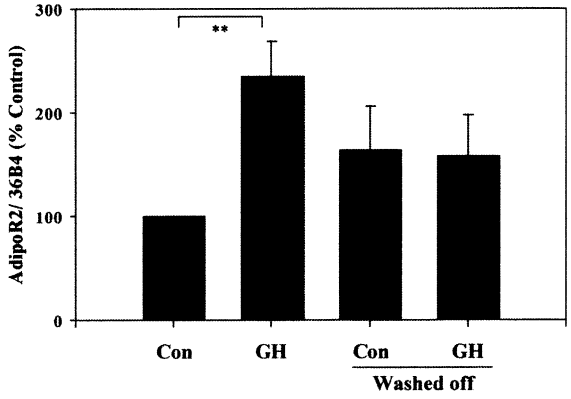

Fig. 5. Stimulation of AdipoR2 expression by GH is reversible. 3T3-L1 adipocytes were serum-starved for 6 h before GH (500 ng/ml) was added for 16 h (columns 1, 2). After this period cells were washed and maintained in culture medium for an additional 24 h (columns 3, 4). Total RNA was extracted and quantitative real-time RT-PCR was performed as described in Section 2. AdipoR2 gene expression normalized to 36B4 mRNA levels is expressed relative to untreated control (Con) cells (=100%). Results are the means ± S.E.M. of three independent experiments. $^{**}P < 0.01$ comparing non-treated with GH-treated adipocytes.

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