Enhancement by Adenosine of Insulin-induced Activation of Phosphoinositide 3-Kinase and Protein Kinase B in Rat Adipocytes*

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The role of adenosine receptor in regulation of insulin-induced activation of phosphoinositide 3-kinase (PI 3-kinase) and protein kinase B was studied in isolated rat adipocytes. Rat adipocytes are known to spontaneously release adenosine, which in turn binds and stimulates the adenosine A<sub>1</sub> receptors on the cells. In the present study, we observed that degradation of this adenosine by adenosine deaminase markedly decreased the insulin-induced accumulation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>), a product of PI 3-kinase. p-Aminophenylacetyl xanthine amine congener (PAPA-XAC), an inhibitor of the adenosine A<sub>1</sub> receptor, also inhibited the insulin-induced PtdIns(3,4,5)P<sub>3</sub> accumulation. When extracellular adenosine was inactivated by adenosine deaminase, phenylisopropyladenosine, an adenosine A<sub>1</sub> receptor agonist, potentiated the insulin-induced accumulation of PtdIns(3,4,5)P<sub>3</sub>. Adenosine deaminase has been shown to impair the insulin sensitivity for glucose transport and antilipolysis by inactivating extracellular adenosine, which adipocytes release spontaneously (1–3). The sensitivity can be restored by treatment of the cells with an adenosine analogue phenylisopropyldenosine (PIA) (4). Because adenosine exerts its effect through the adenosine A<sub>1</sub> receptor coupling to a pertussis toxin-sensitive GTP-binding protein, another activator of the GTP-binding protein, prostaglandin E<sub>2</sub> also affects the insulin sensitivity (5). The effect of adenosine is not accompanied by changes in the cAMP-dependent protein kinase activity and can be observed even when cellular cAMP was maintained at high concentrations (6, 7). Thus adenosine action on the insulin sensitivity is considered to be independent of its inhibitory effect on adenylyl cyclase.

Phosphoinositide 3-kinase (PI 3-kinase), which phosphorylates the 3-OH position of phosphoinositides, is a key signaling enzyme in insulin-induced activation of glucose uptake (8). Studies using either inhibitors of PI 3-kinase such as wortmannin and LY294002 or a dominant negative mutants of PI 3-kinase have demonstrated that PI 3-kinase is necessary for the metabolic action of insulin (9–12). It has also been demonstrated that constitutively active mutants of PI 3-kinase are sufficient to induce the translocation of glucose transporter (GLUT4) to the plasma membrane (13, 14). Although the precise mechanism by which PI 3-kinase regulates the glucose transport system is not completely understood, lipid products of PI 3-kinase, phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) are expected to function as second messengers. In this regard, the lipid products are reported to activate protein kinase B (PKB) directly (15), or indirectly through activation of 3-phosphoinositide-dependent protein kinases (16). Expression of a constitutively activated mutant of PKB has been shown to increase glucose uptake activity of adipose cells (17–19).

Because the mechanism by which adenosine changes insulin sensitivity has not been understood, we examined whether adenosine exerts any effect on insulin-induced activation of PI 3-kinase in rat adipocytes. We observed that adenosine enhanced both the insulin-induced accumulation of PtdIns(3,4,5)P<sub>3</sub> and the insulin-induced activation of PKB by a mechanism independent of its inhibitory action on adenylyl cyclase.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials used in this work were as follows: [32P]<sup>3</sup>P and [γ<sup>32</sup>P]<sup>ATP</sup> from NEN Life Science Products Inc.; p-aminophenylacetyl xanthine amine congener (PAPA-XAC) from Research Biochemicals; 2′,5′-dideoxyadenosine (DDA) from BIOMOL Research Laboratories; (R)-N<sup>6</sup>-(phenylisopropyl)-adenosine (PIA), adenosine deaminase, and histone H2B from Roche Molecular Biochemicals; anti-PKBα(Akt-1) antibody from Santa Cruz; protein G-Sepharose from Pharmacia; and collagenase (type I) from Worthington. Wortmannin was a gift of Dr. Y. Matsuda, Kyowa Hakko Kogyo Co. The reagents for determination of cAMP were kindly supplied by Dr. K. Saito, Yamasa Shoyu Co. All other reagents from commercial sources were of analytical grade.

Isolation of Rat Adipocytes—Epididymal fat pads from male Wistar rats weighing 100–120 g were cut into small pieces and incubated at 37 °C for 30 min in a medium consisting of 130 mM NaCl, 4.7 mM KCl, 6 mM d-glucose...
RESULTS

Suppression by Adenosine Deaminase of Insulin-induced \([32P]PtdIns(3,4,5)P_3\) Accumulation in Rat Adipocytes—Stimulation of the \([32P]P\)-labeled adipocytes with 0.1 \(\mu M\) insulin caused an increased incorporation of radioactivity into a phospholipid fraction corresponding to \(PtdIns(3,4,5)P_3\), a product of PI 3-kinase (Fig. 1). Treatment of the cells with 0.3 \(\mu M\) wortmannin, an inhibitor of the insulin-induced accumulation of \([32P]PtdIns(3,4,5)P_3\), abolished the insulin-induced accumulation of \([32P]PtdIns(3,4,5)P_3\) (Fig. 1). In this experiment, the radioactivity in the \([32P]PtdIns(3,4,5)P_3\) spot from the cells treated with adenosine deaminase was 30.2\% of that from the untreated cells.

Fig. 2A shows typical dose-dependent curves of insulin in the presence or absence of adenosine deaminase. Insulin induced a dose-dependent increase in the \([32P]PtdIns(3,4,5)P_3\) accumulation with the lowest effective concentration around 0.3 nM. Treatment of cells with adenosine deaminase caused a 62 ± 14.5\% decrease in the insulin action (mean ± S.E. from four separate experiments). Half-maximal effects of insulin in the presence or absence of adenosine deaminase were not significantly different. As shown in Fig. 2B, the accumulation of \([32P]PtdIns(3,4,5)P_3\) peaked within 1 min after stimulation with insulin. The increase could still be observed at 10 min after the start of stimulation. The addition of 0.3 \(\mu M\) wortmannin at 1 min after insulin caused a rapid decrease in \([32P]PtdIns(3,4,5)P_3\), indicating a rapid turnover of this lipid product. Adenosine deaminase suppressed the accumulation without causing marked changes in these kinetic properties.

If the inhibitory action of adenosine deaminase is really via degradation of adenosine, prevention of adenosine binding to the \(A_1\) receptor by pharmacological tools is also expected to suppress the insulin action. PAPA-XAC and 8-cyclopentyl-1,3-dipropylxanthine, inhibitors of the adenosine \(A_1\) receptors, inhibited the insulin-induced \([32P]PtdIns(3,4,5)P_3\) production at concentrations of 5 and 10 \(\mu M\), respectively (data not shown, but see Fig. 5 for PAPA-XAC). Thus adenosine deaminase was considered to modulate the insulin-induced accumulation of \([32P]PtdIns(3,4,5)P_3\) by eliminating the adenosine action on the \(A_1\) receptors on the adipocytes.

Enhancement of Insulin-induced \([32P]PtdIns(3,4,5)P_3\) Accumulation by GTP-binding Protein-coupled Receptors—The above results suggested that the spontaneously released adenosine in the absence of adenosine deaminase or the \(A_1\) receptor antagonists potentiated the insulin action on the accumulation of \([32P]PtdIns(3,4,5)P_3\). Thus we next intended to directly show that stimulation of the \(A_1\) receptors potentiates the insulin-induced accumulation of \([32P]PtdIns(3,4,5)P_3\) under the condition where the extracellular adenosine was depleted. For this purpose, PIA, a poorly hydrolyzable analogue of adenosine, was
utilized. When the spontaneously released adenosine was inactivated by adenosine deaminase, PIA effectively enhanced the insulin-induced \([^{32}P]PtdIns(3,4,5)P_3\) production in a dose-dependent manner (Fig. 3). The effect of PGE\(_2\) on the insulin-induced \([^{32}P]PtdIns(3,4,5)P_3\) accumulation was examined in the absence and presence of various concentrations of insulin. Adenosine deaminase was preincubated for 30 min with or without 2 units/ml adenosine deaminase and then for 5 min in the presence or absence of 0.1 \(\mu M\) insulin. After extraction and separation of \([^{32}P]PtdIns(3,4,5)P_3\), the radioactivity in the lipid fraction was determined using a Fuji BAS2000 analyzer. The results are shown as percent of the radioactivity in the PtdIns(3,4,5)P\(_3\) fraction from the cells treated with 0.1 \(\mu M\) insulin alone for 5 min. Each point indicates the mean ± S.E. from three separate experiments.

In the experiment shown in Fig. 5, the effect of endogenous adenosine was prevented by the addition of 5 \(\mu M\) PAPA-XAC, an inhibitor of adenosine A\(_1\) receptors. DDA, which directly inhibits adenylyl cyclase without activating GTP-binding protein, also attenuated the insulin-induced \([^{32}P]PtdIns(3,4,5)P_3\) accumulation. In contrast, direct inhibition of adenyl cyclase by DDA did not affect the accumulation of the lipid product even at high concentrations.

One possible explanation for the failure of DDA to increase

\[\text{Adenosine Action on PI 3-Kinase}\]

\[\text{FIG. 3. Synergistic stimulation of } [^{32}P]PtdIns(3,4,5)P_3 \text{ accumulation by insulin and PIA. Isolated adipocytes, labeled with } [^{32}P], \text{ were incubated at } 37\text{ °C for } 5\text{ min with or without } 2\text{ units/ml adenosine deaminase for } 5\text{ min with the indicated concentrations of PIA, and then for } 5\text{ min in the presence or absence of } 0.1\mu M\text{ insulin. After extraction and separation of } [^{32}P]PtdIns(3,4,5)P_3, \text{ the radioactivity in the lipid fraction was determined using a Fuji BAS2000 analyzer. The results are shown as percent of the radioactivity in the PtdIns(3,4,5)P}_3 \text{ fraction from the cells treated with } 0.1\mu M\text{ insulin alone for } 5\text{ min. Each point indicates the mean ± S.E. from three separate experiments.}\]
In the present study, we showed that activation of a pertussis toxin-sensitive GTP-binding protein enhanced the insulin-induced incorporation of radioactive into the fraction of PtdIns(3,4,5)P_3 in PtdIns(3,4,5)P_3-labeled rat adipocytes. The increased incorporation is considered to accompany the increased intracellular concentration of the second messenger because insulin-
induced activation of PKB, the activity of which is under the
control of the lipid product of PI 3-kinase, was also enhanced
by activation of the GTP-binding protein. Thus, the receptors cou-
pling to the GTP-binding protein were found to positively reg-
ulate insulin-signaling systems by affecting the production of
the second messenger of insulin.

It is reported that adenosine modulates the insulin action for
glucose transport in adipocytes (2, 3). Adenosine exerts this
effect through the adenosine A1 receptor coupling to a pertussis
toxin-sensitive GTP-binding protein. Although the GTP-bind-
ing protein is a negative regulator of adenyl cyclase, the aden-
osine effect on the insulin action has been regarded as
mostly independent of the change in the adenyl cyclase ac-
tivity (6, 7). In the present paper, we showed that adenosine
has an ability to enhance the insulin-signaling cascade medi-
ated by PI 3-kinase. This effect of adenosine was through the
GTP-binding protein but was not due to the decreased produc-
tion of cAMP, because the direct inhibition of adenyl cyclase
did not affect the insulin actions on PtdIns(3,4,5)P3 and PKB.
Thus the enhanced accumulation of PtdIns(3,4,5)P3 is another
cAMP-independent effect of adenosine. Recent studies dem-
onstrated that PI 3-kinase is enough to stimulate the transloca-
tion of glucose transporter from intracellular compartment to
plasma membrane (13, 14). Constitutively activated mutants of
PKB is shown to cause the transporter translocation (17–19).
Thus it is intriguing to discuss a possible effect of the enhanced
PtdIns(3,4,5)P3 accumulation on the cellular glucose transport
activity.

Fig. 6 shows a correlation between a [32P]PtdIns(3,4,5)P3 con-
tent and a rate of glucose uptake in insulin-treated rat
adipocytes. The open symbols represent the results obtained
when the cells were treated with the various concentrations of
insulin alone. The results from the cells treated with the sub-
maximal concentrations (0.01–0.3 μM) of wortmannin and 0.1
μM insulin were shown by the closed symbols. The correlation
indicates that the rate of glucose uptake is saturated by a small
change in the [32P]PtdIns(3,4,5)P3 content. Because of this
saturation characteristic, an enhanced accumulation of the sec-
ond messenger is expected to cause the increased glucose
uptake only when the cells are stimulated by submaximal
concentrations of insulin. Previous studies in literature indi-
cated that adenosine increases both insulin sensitivity and
maximal response to insulin for glucose transport. One report
showed that adenosine increases the glucose uptake in re-
sponse to a maximal concentration of insulin without changing
the extent of the translocation (6). Thus the augmented pro-
duction of PtdIns(3,4,5)P3 is not considered to explain the effect
of adenosine on the maximal glucose uptake but may contrib-
ute in some degree to the increased insulin sensitivity for

![Fig. 6. Correlation between hexose uptake and PtdIns(3,4,5)P3 accumulation. Isolated adipocytes were incubated at 37 °C for 10 min with various concentrations of insulin. The cells were then measured for the [32P]PtdIns(3,4,5)P3 accumulation. Open circles indicate the results obtained when the cells were treated with various concentrations (0–0.1 μM) of insulin alone, while the closed circles were the results obtained when the cells were treated with various concentrations (0.01–0.3 μM) of wortmannin before treat-
iment with 0.1 μM insulin. Each point indicates the mean from two to
five separate experiments.](image-url)
3-kinase by recruiting the enzyme to membrane receptors (instead of inducing the association with cytosolic protein IRS-1). In order to address this point, we examined the effect of PDGF on rat adipocytes, because stimulation of PDGF receptors on the cells is reported to increase the PI 3-kinase activity associated with an antibody against the receptors even when the cells have not been transfected with the receptors (26). However, we could not detect any effect of PDGF on the PtdIns(3,4,5)P3 production and the PKB activation regardless of whether adenosine deaminase is included in the assay mixture (data not shown). Thus further study using other cell systems is necessary and is now under study in our laboratory to clarify if the interaction could be observed for the agonists other than insulin.

In summary, the present paper showed that a second messenger production by insulin is positively regulated by the receptors coupling to GTP-binding protein in physiological target cells of insulin. Although the precise molecular mechanism of this cross-talk is not clear now, the finding is intriguing in view of the central role of PI 3-kinase in insulin-signaling cascades leading to metabolic and mitogenic cellular responses. One possible consequence of the cross-talk is a modified insulin sensitivity for glucose transport as discussed above. In this regard, it is interesting to note that the abnormal function of adenosine A1 receptors in genetically obese animals and the decreased level of GÎ³ in a streptozotocin-induced diabetic rat have been reported (27, 28). Furthermore, a study using transgenic mice has demonstrated that a deficiency of GÎ³2 produces impaired glucose tolerance and resistance to insulin (29). A possible relation of the present study to these observations is intriguing to be examined.

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