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Mechanism of O_2^-- and H_2O_2-induced stimulation of sugar transport in mouse fibroblast BALB/3T3 cells

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Xanthine/xanthine oxidase and H_2O_2 stimulated sugar transport. Application of superoxide dismutase and catalase to the cells showed an inhibitory effect on these agent-stimulated sugar transports. Addition of amiloride and 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), which abolish the cytoplasmic alkalinization, inhibited the stimulation of sugar transport by xanthine/xanthine oxidase in the presence of catalase. The calmodulin antagonists, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and trifluoperazine inhibited H_2O_2-stimulated sugar transport. These results suggest that O_2^- stimulates sugar transport in an intracellular pH-dependent manner and that H_2O_2 stimulates sugar transport in a calcium-calmodulin-dependent manner. These mechanisms may be involved in sugar-transport stimulation in mouse fibroblast BALB/3T3 cells by the tumor-promoting phorbol ester phorbol-12,13-dibutyrate and insulin, since the stimulatory effects of these agents were inhibited by scavengers of oxygen radicals.

Introduction

The superoxide radical anion (O_2^-), which is able to initiate a radical chain reaction [1], is a common intermediate in the univalent reduction of oxygen. In contrast, hydrogen peroxide (H_2O_2) is formed by bivalent reduction of oxygen or dismutation of O_2^-. H_2O_2 and O_2^- have been shown to be implicated as toxic agents when cells are exposed to externally generated oxygen radicals. A number of oxidants have been known to stimulate hexose monophosphate shunt activity, and H_2O_2 was shown to stimulate glucose uptake [2,3], glucose oxidation [4], and glucose incorporation into glycogen [5].

O_2^- release and/or H_2O_2 production is known to be stimulated by phorbol-12,13-dibutyrate and insulin [6–10]. This phenomenon is one of the earliest responses detected after treatment with these agents. Recently, it was reported that serum-, phorbol-12,13-dibutyrate- and xanthine/xanthine oxidase-induced O_2^- release, but not H_2O_2 release, caused an increase in the intracellular pH in leucocytes [11], and that this phenomenon was inhibited by vitamin E or superoxide dismutase. On the other hand, H_2O_2 mediates calcium release from mitochondria [12,13]. Intracellular pH and intracellular calcium are important as a secondary messenger in signal-coupling responses [14], and our previous studies showed that these messengers

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Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperadine.

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regulate sugar-transport activity in mouse fibroblast Swiss 3T3 cells [15,16].

Xanthine/xanthine oxidase is one of the enzyme systems generating O_2^- and H_2O_2 [17,18]. This enzyme and its mechanism of action have been thoroughly investigated [19–21], and have been frequently used as a model system to investigate many cellular reactions initiated by O_2^- and H_2O_2. In this paper, we studied the action mechanisms of O_2^- and H_2O_2 on the sugar-transport system in mouse fibroblast BALB/3T3 cells, using the xanthine/xanthine oxidase system and comparing it with those of phorbol-12,13-dibutyrate and insulin.

Materials and Methods

Cell culture. BALB/3T3/A31 cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum at 37° C. The cells were maintained at the subconfluent state by passage every 2–3 days. Cells from stock cultures were trypsinized and seeded ($3 \cdot 10^5$ cells per 2 ml) into tissue culture dishes (35 mm in diameter). The cultures were incubated at 37° C in a water-saturated atmosphere containing 5% CO_2. 3–4 days later, monolayer cells that had become confluent were used for the experiments.

Measurement of 2-deoxyglucose uptake by intact cells. Cells were incubated with or without the test compound for a designated period of time, and rinsed twice with 2 ml of phosphate-buffered saline (pH 7.4). The uptake was initiated by the addition of 0.8 ml of phosphate-buffered saline containing 2-deoxyglucose (4 μM, 0.1 μCi/ml) at 20° C. After 2 min, the 2-deoxyglucose uptake was stopped by washing the plates three times with 2 ml of ice-cold phosphate-buffered saline. The cells were denatured by addition of 0.1 ml of 5% trichloroacetic acid and then dissolved in 0.9 ml of 0.1 M NaOH/0.1% sodium dodecyl sulfate, and aliquots of the lysate were taken for radioactivity assay and protein determination. Carrier-mediated uptake was determined after correction with L-glucose. Transport of 2-deoxyglucose (4 μM) in BALB/3T3/A31 cells was linear with time for at least 10 min and 2-deoxyglucose equilibrated across the membrane in 3–5 min.

Materials. 2-Deoxy[^{3}H]glucose (5 Ci/mmol) and L-[^{3}H]glucose (10.7 Ci/mmol) were purchased from New England Nuclear. Fetal calf serum was purchased from Irvine Scientific. Phorbol-12,13-dibutyrate was obtained from P-L Biochemicals Inc. and xanthine oxidase was from Boehringer-Mannheim. W-7 and 1-(5-isoquinolinesulfonyl)2-methylpiperadine (H-7) were from Seikagaku Kogyo Co.; amiloride and SITS were from Sigma; insulin, vitamin E, trifluoperazine, superoxide dismutase and catalase were from Nakarai Chemical Co. All other chemicals were obtained from commercial sources and were either reagent grade or of the highest purity available.

Results and Discussion

We examined the effect of O_2^- and H_2O_2 generated by xanthine/xanthine oxidase on the sugar transport system. Fig. 1 shows that xanthine/xanthine oxidase as well as H_2O_2 stimulates 2-deoxyglucose uptake time-dependently and the stimulation was observed soon after addition of these agents. The maximal response (2.5-fold) was

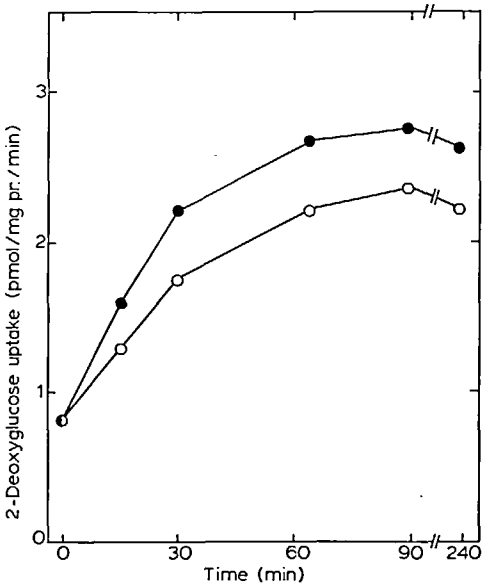

Fig. 1. Effect of xanthine/xanthine oxidase or H_2O_2 on 2-deoxyglucose uptake. Cells were treated for the designated period of time with xanthine (100 μM)/xanthine oxidase (0.1 U/ml) (○) or H_2O_2 (150 μM) (●) and then the 2-deoxyglucose uptake was assayed for 2 min. Each point represents the mean ± S.D. ($n = 5$).

TABLE I

EFFECT OF O_2^- AND/OR H_2O_2 SCAVENGERS ON XANTHINE/XANTHINE OXIDASE- OR H_2O_2-STIMULATED SUGAR TRANSPORT

The cells were incubated in serum-free Eagle's minimum essential medium in the presence of vitamin E or superoxide dismutase and/or catalase for 10 min and then either xanthine (100 μM)/xanthine oxidase (0.1 U/ml) or H_2O_2 (150 μM) was added. After 60 min, 2-deoxyglucose uptake was assayed for 2 min. Data are expressed as mean ±S.D. ($n = 5$).

Addition	2-Deoxyglucose uptake (pmol/mg protein per min)		
	none	xanthine/ xanthine oxidase	H_2O_2
None	1.02±0.08	2.15±0.13 *	2.58±0.23 *
Vitamin E (25 μM)	0.91±0.07	1.08±0.09	1.17±0.11
Superoxide dismutase (100 U/ml)	0.92±0.09	2.36±0.18 *	2.61±0.22 *
Catalase (1000 U/ml)	0.90±0.06	2.03±0.17 *	1.07±0.10
Superoxide dismutase +catalase	0.84±0.07	0.90±0.07	0.92±0.08

* $P < 0.05$, vs. control value.

achieved after 90 min, with a half-maximal time of approx. 20 min, and it was maintained up to 4 h in the presence of xanthine/xanthine oxidase or H_2O_2. These stimulatory effects were reversed by removal of the agents (data not shown). Moreover, the 3-*O*-methylglucose (non-metabolized analogue of D-glucose) uptake was stimulated by xanthine/xanthine oxidase or H_2O_2 to the same degree as that measured by 2-deoxyglucose (data not shown). This result suggests that the changes in transport may be responsible for the alteration of sugar transport.

Table I shows the effect of vitamin E, superoxide dismutase, catalase or superoxide dismutase plus catalase on xanthine/xanthine oxidase- or H_2O_2-stimulated sugar transport. Both xanthine/xanthine oxidase- and H_2O_2-stimulated sugar transports were suppressed by vitamin E. H_2O_2-stimulated sugar transport was inhibited by catalase but not by superoxide dismutase. Xanthine/xanthine oxidase-stimulated sugar transport was neither inhibited by superoxide dismutase nor catalase alone. However, elimination of O_2^- and H_2O_2 from the reaction mixture by addition of both superoxide dismutase and catalase inhibited the stimulation of sugar transport by xanthine/xanthine oxidase. These results clearly indicate that the stimulation of sugar transport is dependent on O_2^- and/or H_2O_2.

Intracellular pH is known to be primarily regulated by a Na^+/H^+ exchanger and a Na^+-dependent HCO_3^-/Cl^- exchanger, and inhibited by amiloride and SITS, respectively [22–24]. Table II shows that xanthine/xanthine oxidase-stimulated sugar transport was inhibited about 50% in the presence of amiloride and SITS. Although this stimulation was not inhibited by catalase alone as described above (Table I), it was completely inhibited by amiloride and SITS in the presence of catalase. These results suggest that O_2^- stimulates sugar transport by activation of Na^+/H^+ exchanger and/or HCO_3^-/Cl^- exchanger resulted in the alkalinization of the cytoplasm. On the other hand, H_2O_2-stimulated sugar transport was not affected by amiloride and SITS. This suggests that H_2O_2 stimulates the sugar transport independently of intracellular pH.

TABLE II

EFFECT OF AMILORIDE AND SITS ON THE XANTHINE/XANTHINE OXIDASE- OR H_2O_2-STIMULATED SUGAR TRANSPORT

The cells were incubated in serum-free Eagle's minimum essential medium in the presence of amiloride (50 μM) and SITS (50 μM) and/or superoxide dismutase (100 U/ml) and/or catalase (100 U/ml) for 10 min and then xanthine (100 μM)/xanthine oxidase (0.1 U/ml) or H_2O_2 (150 μM) was added. After 60 min, 2-deoxyglucose uptake was assayed for 2 min. Data are expressed as mean±S.D. ($n = 5$).

Addition	2-Deoxyglucose uptake (pmol/mg protein per min)		
	none	xanthine/ xanthine oxidase	H_2O_2
None	1.10±0.06	2.03±0.18 *	2.47±0.20 *
Amiloride+SITS	1.16±0.09	1.55±0.20	2.59±0.28 *
Amiloride+SITS +superoxide dismutase	1.01±0.11	1.79±0.19 *	2.44±0.31 *
Amiloride+SITS +catalase	0.87±0.09	0.98±0.10	–

* $P < 0.05$, vs. control value.

TABLE III

EFFECT OF W-7, TRIFLUOPERAZINE OR H-7 ON THE XANTHINE/XANTHINE OXIDASE- OR H_2O_2-STIMULATED SUGAR TRANSPORT

The cells were incubated in serum-free Eagle's minimum essential medium in the presence of W-7, trifluoperazine or H-7 for 10 min and then xanthine (100 μM)/xanthine oxidase (0.1 U/ml) or H_2O_2 (150 μM) was added. After 60 min, 2-deoxyglucose uptake was assayed for 2 min. Data are expressed as mean ± S.D. ($n = 5$).

Addition	2-Deoxyglucose uptake (pmol/mg protein per min)		
	none	xanthine/ xanthine oxidase	H_2O_2
None	1.03 ± 0.05	2.08 ± 0.20 *	2.51 ± 0.20 *
W-7 (50 μM)	0.86 ± 0.05	1.91 ± 0.20 *	1.10 ± 0.08
Trifluoperazine (100 μM)	0.88 ± 0.06	2.01 ± 0.17 *	1.07 ± 0.06
H-7 (50 μM)	1.05 ± 0.08	2.11 ± 0.16 *	2.55 ± 0.18 *

* $P < 0.05$, vs control value.

Since it has been reported that H_2O_2 mediates calcium release from mitochondria [12,13] and that the stimulation of sugar transport by H_2O_2 was blocked by chelation of intracellular calcium [3], H_2O_2 appeared to involve a calcium-dependent pathway. Therefore, the effect of H-7, W-7 and trifluoperazine on H_2O_2-stimulated sugar transport were examined. It has been known that H-7 inhibits calcium-activated, phospholipid-dependent protein kinase more specifically than other kinases [25], and that W-7 and trifluoperazine are calmodulin antagonists [26]. As shown in Table III xanthine/xanthine oxidase-stimulated sugar transport was not inhibited by these agents, whereas H_2O_2-stimulated sugar transport was inhibited by W-7 and trifluoperazine but not by H-7. These results suggest that the activation of calcium-calmodulin is involved in the stimulation of sugar transport by H_2O_2.

Tumor promoting phorbol esters induce O_2^- release of which activity is closely correlated with their tumor promoting activity [6,7,27]. Insulin is known to stimulate intracellular H_2O_2 production, which mediates some of the effect of insulin [8,9]. The pretreatment of cells with several antioxidants, such as butyl-4-hydroxyanisole, curcumin, gallic acid, rutin and vitamin E, resulted in the inhibition of sugar transport stimulated by phorbol-12,13-dibutyrate or insulin by 60–80% (Fig. 2). Moreover, the pretreatment of cells with

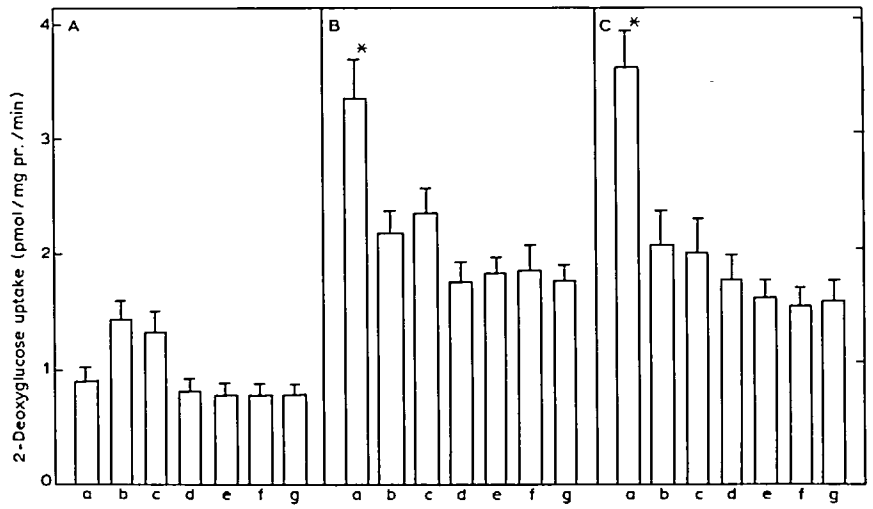

Fig. 2. Effect of antioxidants on phorbol-12,13-dibutyrate- or insulin-stimulated 2-deoxyglucose uptake. Confluent cells were incubated in serum-free Eagle's minimum essential medium in the absence (a), or presence of (b) butyl-4-hydroxyanisole (150 μM), (c) curcumin (5 μM), (d) gallic acid (150 μM), (e) rutin (150 μM), (f) vitamin E or (g) superoxide dismutase (100 U/ml) plus catalase (1000 U/ml) for 10 min. Then, either (B) phorbol-12,13-dibutyrate (10 nM) or (C) insulin (20 μg/ml) was added to the medium and the incubation continued for 30 min. 2-Deoxyglucose uptake for 2 min was assayed. Data are expressed as mean ± S.D. ($n = 5$). (* $P < 0.05$, compared to corresponding values which were obtained in the presence of each antioxidant.)

superoxide dismutase plus catalase, O_2^- and H_2O_2 scavenger, resulted in the inhibition of sugar transport stimulated by these agents. On the other hand, superoxide dismutase or catalase alone did not inhibit these stimulations (data not shown). These results suggest that O_2^- release and/or H_2O_2 production by phorbol ester and insulin may be important in the stimulation of sugar-transport activity. Moreover, phorbol-12,13-dibutyrate- and insulin-stimulated sugar transport was completely blocked by amiloride, SITS and W-7 (data not shown).

Czech et al. [2,4,28] proposed that insulin activation of fat-cell sugar transport involved the oxidation of certain sulfhydryls to the disulfide form. The sugar transport system or a regulatory component of the system was hypothesized to exist in an active reduced form which is capable of promoting sugar transport. In support of the model, Czech et al. found that oxidants with a high affinity for sulfhydryls such as H_2O_2 and diamine mimicked the stimulatory effect of insulin on sugar transport while reductants inhibited transport activity [2]. It seems that the key membrane sulfhydryls are associated with either the signal that transmits insulin action or the transport system itself that responds to the signals.

Recently it was reported that insulin action on sugar transport represented an additive effect on transporter translocation and intrinsic transporter activity in fat cells [29–31]. However, the action of intrinsic activity of sugar transporter was not observed in mouse fibroblasts [16,32]. Our previous studies showed that phorbol-12,13-dibutyrate- or insulin-stimulated sugar transport in mouse fibroblast Swiss 3T3 cells is regulated at least three different mechanisms [15,16,32,33]; (i) calcium-calmodulin-dependent regulation, (ii) intracellular pH-dependent regulation, and (iii) calcium-activated phospholipid-dependent protein kinase-dependent regulation. These mechanisms lead to the translocation of sugar transporters from an intracellular sites to the plasma membrane; this process results in an increase in the rate of sugar transport. Combining these previous observations with the present results, we have concluded that the calcium-calmodulin-dependent process in H_2O_2-stimulated sugar transport system and intracellular pH-dependent process in O_2^--stimulated sugar transport system may be involved in the essential steps for the stimulation of sugar transport in mouse fibroblast cell by phorbol-12,13-dibutyrate and insulin.

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