Endothelin Mobilizes Calcium and Enhances Glucose Uptake in Cultured Human Skeletal Myoblasts and L6 Myotubes

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Abstract  In this study we used endothelin as a paradigm to explore the concept that some vasoactive agents, acting through mobilization of Ca\(^{2+}\) and stimulation of protein kinase C, can interact with human skeletal muscle and modify its glucose transport. Cultured human skeletal myoblasts from the vastus lateralis demonstrated two subclasses of high-affinity endothelin receptors and a robust increase in cytosolic free Ca\(^{2+}\) upon exposure to endothelin. The endothelin-evoked rise in cytosolic free Ca\(^{2+}\) primarily resulted from Ca\(^{2+}\) mobilization from intracellular organelles. Both endothelin and insulin enhanced \([\text{H}]\)deoxy-d-glucose uptake in human myoblasts, but their effects were not additive. These findings also were observed in differentiated myotubes of L6 skeletal muscle cells. Moreover, \([\text{H}]\)deoxy-d-glucose uptake in human myoblasts was enhanced by treatment with phorbol 12-myristate 13-acetate. The endothelin- and insulin-mediated increases in \([\text{H}]\)deoxy-d-glucose were totally ablated by treatment with calphostin C. Such observations suggest that endothelin can enhance glucose uptake in human skeletal muscle. This is mediated through mechanisms that are at least partially protein kinase C dependent. Thus, increased levels of endothelin in vascular beds may contribute to altered glucose metabolism in essential hypertension. (Hypertension. 1994;23[part 2]:1075-1081.)

Key Words • receptors, endothelin • protein kinase C • calcium ions • hypertension, essential • glucose

Essential hypertension is marked by insulin resistance resulting from altered glucose metabolism in skeletal muscles.\(^1\)-\(^4\) Altered glucose metabolism may relate to hemodynamic factors, including circulating vasoactive agents responsible for blood flow distribution between insulin-sensitive and insulin-insensitive tissues.\(^5\) Alternatively, it could reflect perturbations in the immediate environment of skeletal muscles that are caused by a dysfunctional vascular endothelium. The vascular endothelium plays a central function in regulating vascular tone through the release of vasoactive agents.\(^6\),\(^7\) Thus, an imbalance between vasoconstrictors and vasodilators of endothelial origin could provoke peripheral vasoconstriction, increased peripheral vascular resistance (PVR), and hypertension.\(^7\),\(^8\) We postulate that a number of endothelial factors, acting on vascular smooth muscle cells (VSMCs), also interact with skeletal muscle cells to alter glucose metabolism through the same cellular pathways that promote the increase in the PVR. The action of endothelin (ET), a potent vasoconstrictor of endothelial origin,\(^9\)-\(^11\) was used to test this hypothesis.

Although the systemic infusion of ET can produce hypertension in the rat,\(^1\)\(^2\) a controversy exists as to whether the circulating levels of this factor are increased in essential hypertension. Moreover, it is uncertain whether elevated levels of ET cause hypertension in humans (reviewed in Reference 7). It may well be that the plasma levels of ET reflect a spillover from a variety of sites and that the main effects of this factor are exerted through its paracrine or autocrine function.\(^1\)\(^3\) ET production and secretion\(^1\)\(^3\)-\(^16\) and ET-specific receptors\(^1\)\(^7\),\(^18\) have been demonstrated in a number of cells and tissues in addition to the endothelium and vascular smooth muscle. As shown herein, ET receptors also are present in cultured human skeletal myoblasts (SKMBs). Thus, ET may interact in situ not only with cellular elements in the vascular wall but also with skeletal muscle cells in the immediate proximity of vascular beds. It is this interaction with skeletal muscle of vasoactive agents such as ET that in the final analysis could provide a common thread for altered glucose metabolism and increased PVR in essential hypertension.

The aforementioned concepts are particularly attractive because a number of vasoactive agents originating in the endothelium, including ET, act through raising the cytosolic Ca\(^{2+}\), and stimulating protein kinase C (PKC).\(^1\)\(^9\)-\(^2\) Since increased PKC activity\(^2\)\(^3\),\(^2\) and perhaps a rise in Ca\(^{2+}\) can both enhance glucose uptake and blunt insulin action in some tissues, the activation of this enzyme in skeletal muscle through Ca\(^{2+}\)-dependent or Ca\(^{2+}\)-independent pathways may hold the key for insulin resistance in essential hypertension (reviewed in Reference 26). This tenet was explored by examining the effect of ET on cultured human SKMBs and on differentiated myotubes derived from L6 cells.

Methods

Preparations of Cultured SKMBs

Biopsies of skeletal muscle were obtained from the vastus lateralis of 10 subjects (6 men, 4 women) with no neuromuscular or metabolic diseases who underwent surgical procedures unrelated to the purpose of this study. Informed consent approved by the Institutional Review Board was obtained in all cases. Specimens were rinsed three times and finely chopped.
in penicillin containing α-minimum essential medium (α-MEM) (30 U/mL) and streptomycin (30 μg/mL). The tissue was trypsinized for 60 minutes at 37°C with 0.01% gaseous collagenase (Worthington Biochemicals), 0.01% gaseous trypsin inhibitor (Sigma), and 0.03% gaseous bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4). Tissue was passed three to five times through a 26-gauge needle and centrifuged for 8 minutes at 200g. The pellet was suspended in α-MEM, 15% fetal bovine serum (FBS) plus 2 mmol/L L-glutamine, 10 μg/mL penicillin, and 10 μg streptomycin per milliliter. Cells were propagated in 75-cm² flasks for 2 to 3 weeks (medium changes every 3 days). The α-MEM was then replaced by Dulbecco's modified Eagle medium (DMEM) plus 10% FBS, 2 mmol/L L-glutamine, and no antibiotics. Purity of the preparations was ascertained by immunohistochemical staining with anti-muscle actin-specific monoclonal antibody HHF35 (Enzo Diagnostics).

In preparation for cell seeding, confluent layers were treated for 2 minutes at room temperature with trypsin-EDTA mixture (0.25% and 0.1%, respectively, JRH Biosciences). The reaction was stopped by the addition of DMEM plus 10% FBS. Cells were seeded either into 6-well (35-mm diameter) clusters (= 50 0000 cells per well in 8 mL of medium) or 24-well (16-mm diameter) plates (100 to 150 000 cells per well in 2 mL of medium). Each well contained a 13.8 x 30-mm glass coverslip. Cells were grown in the respective wells for 3 to 4 days until subconfluent. Unless indicated, the medium was replaced with DMEM plus 0.1% FBS for the subsequent 24 hours. The DMEM was supplemented with 20 mmol/L glucose for cell preparations designated for studying [3H]deoxy-o-glucose ([3H]DOG) uptake. Passages 2 through 6 were studied. In preliminary studies we demonstrated no effect of passage number or the sex of the donor of the SKMBs on the response of the cells to ET.

Preparations of Myotubes From L6 Muscle Cells

L6 muscle cells originating from rat skeletal muscle were obtained from A. Klip (Hospital for Sick Children, Toronto, Canada). Conditions for differentiation of myotubes were maintained as previously described. Briefly, L6 cells were grown in α-MEM plus 2% FBS and antibiotics in 75-cm² flasks until confluent. Cells then were seeded into either 24-well plates (for [3H]DOG uptake measurements) at a density of 70 0000 cells per well or 6-well clusters containing glass coverslips (for Ca²⁺ monitoring) at a density of 210 000 cells per well. Morphological differentiation into myotubes was observed in approximately 10 days, and cells were studied on the 13th day after seeding. Medium was changed every 72 hours. In preparation for experiments, myotubes were maintained in serum-free α-MEM for 24 hours. Medium was then replaced with fresh serum-free α-MEM supplemented with 20 mmol/L glucose for an additional 6 hours. Morphological determination of myotube formation was ascertained after fixation with glutaraldehyde using 0.4% Giemsa stain. Viability, determined using trypan blue exclusion, was greater than 95%.

Ca²⁺ Measurements

These measurements were performed essentially as described before. Briefly, coverslips containing subconfluent layers of SKMBs or L6 myotubes were rinsed with HEPES-buffered solution (HBS) comprising (in mmol/L) NaCl 130, KCl 5, MgCl₂ 1, CaCl₂ 1.8, HEPES 20, and glucose 5 (pH 7.4). They were then incubated for 40 minutes at room temperature in the HBS plus 2.5 mmol/L (Molecular Probes). The cells were then either preincubated in the HBS for an additional 10 minutes at 37°C. Slides were sealed in cassettes in a SPEX CM3 fluorescence spectrometer (SPEX Industries, Inc) equipped with a thermostatically controlled cell holder, a stirrer, and a suction device to remove solutions. The resting Ca²⁺ was monitored for 1 to 2 minutes. The agonists (ET-1 and angiotensin II [Ang II]) were added thereafter. Excitation and emission wavelengths were set at 430/380 and 505 nm, respectively.

ET Binding Experiments in Human SKMBs

In preliminary experiments we ascertained that the binding of [125I]-ET-1 (NEX-259, New England Nuclear) reached a plateau within 240 minutes of incubation at 21°C. All subsequent saturation binding experiments were performed by incubating cells in 24-well plates at 21°C for 4 hours. Nonspecific bindings were determined in the presence of 400 nmol/L of unlabeled ET-1 (Calbiochem). Cell numbers in the wells were determined by a Coulter counter.

[3H]DOG Uptake in Human SKMBs and L6 Myotubes

Cells in 24-well plates were preincubated at 37°C for 60 minutes in culture medium with or without different concentrations of paracrine insulin (Sigma) with or without ET or with their combinations. In preliminary studies we showed that for human SKMBs, maximal stimulatory doses for insulin and ET were 500 and 100 nmol/L, respectively. For measuring the effect of 500 nmol/L phorbol 12-myristate 13-acetate (PMA), the phorbol ester was added at the last 5 minutes of preincubation in DMEM. In experiments examining the effect of 50 nmol/L calphostin C, the agent was included for the last 20 minutes of preincubation. Cells were rinsed once with glucose-free HBS, and 0.5 mL of fresh HBS containing 10 μmol/L [3H]DOG (New England Nuclear) with or without the aforementioned agonists was added to each well. The uptake experiments were performed at 37°C for 15 minutes for SKMBs and 10 minutes for L6 myotubes after establishing in preliminary studies the linearity of this process within these periods of incubation. At the end of each uptake experiment, cells in each well were rinsed three times with 2 mL aliquots of ice-cold HBS and treated with 0.1N NaOH overnight. The cells were then scraped and counted in a scintillation counter. Noncarrier-mediated [3H]DOG uptake was determined in the presence of 5 μmol/L cytochalasin B and was subtracted from all other uptake values. There was no apparent effect of the vehicle for PMA (0.1% dimethyl sulfoxide) on [3H]DOG uptake. Protein measurements used the Lowry method. Each [3H]DOG measurement was performed in triplicate. Statistical analyses to determine significance among the various treatments used analysis of variance for repeated measures (SAS Institute).

Results

Human SKMBs

ET evoked Ca²⁺ responses in 1.8 mmol/L Ca²⁺ HBS (Fig 1A) and Ca²⁺-free HBS (CaCl₂ removed and 0.3 mmol/L EGTA added; Fig 1B). The Ca²⁺ responses could be demonstrated at an ET concentration as low as 1 nmol/L. However, typical agonist-evoked Ca²⁺ transients were evident at concentrations of 5 nmol/L or above. There were no apparent differences in the magnitude of the peak Ca²⁺ transients between cells treated in Ca²⁺-free or Ca²⁺-containing HBS, although the posttransient Ca²⁺ levels were slightly higher in the Ca²⁺-containing medium (Fig 1A and 1B). Similar results were obtained in nonquiescent SKMBs that were not deprived of FBS 24 hours before the Ca²⁺ measurements (not shown). These observations differ from those in cultured human fibroblasts, myometrial cells, and VSMCs, in which ET-evoked transients were substantially higher in Ca²⁺-containing than in Ca²⁺-free medium. The ET-evoked Ca²⁺ response of the SKMBs also differed from that of smooth muscle cells by its insensitivity to the Ca²⁺ channel blockers verapamil, nicardipine, and diltiazem (Fig 1C). This finding is not surpris-
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Figure 1. Graphs show effect of endothelin (ET) on the Ca\(^{2+}\) profile in cultured human skeletal myoblasts (SKMBs). A, Dose response of ET effect in 1.8 mmol/L Ca HEPES-buffered solution (HBS); B, effect of 50 nmol/L ET on the Ca\(^{2+}\) profile in SKMBs in 1.8 mmol/L Ca-containing HBS (+Ca\(^{2+}\)) and Ca-free HBS (-Ca\(^{2+}\)); and C, lack of effect of Ca channel blockers on the ET (50 nmol/L)-evoked Ca\(^{2+}\) increase. a indicates no Ca channel blockers; b, 0.3 μmol/L nicardipine; c, 10 μmol/L verapamil; and d, 10 μmol/L diltiazem. Arrows denote addition of ET to the medium. Vertical spikes represent SEM. SKMBs for experiments depicted under A, B, and C were from 4, 3, and 3 subjects, respectively.

Because the main effect of ET was exerted through Ca\(^{2+}\) mobilization from intracellular organelles, whereas external calcium entry serves to replenish these stores.

In further experiments, we demonstrated the presence of specific ET receptors on the surface of these cells (Fig 2). Scatchard analysis of the binding data suggests the presence of distinct subclasses of receptors, one with \(B_{\text{max}}=14,241\) receptors per cell, \(kD=0.12\) nmol/L, and the other with \(B_{\text{max}}=20,720\) receptors per cell, \(kD=0.41\) nmol/L. These values are consistent with findings of others using different cells.29-31

Next we measured glucose uptake in SKMBs, using \([\text{H}]\)DOG (Fig 3A). In the absence of insulin, \([\text{H}]\)DOG uptake was 31.0±2.7 pmol/mg protein per minute and increased by 41.1% to 43.8±3.6 pmol/mg protein per minute after treatment with maximal insulin concentration (500 nmol/L). These findings in subconfluent human SKMBs from the vastus lateralis are quite similar to those observed in fused myotubes originating from the human gracilis muscle.32 Furthermore, ET (at a maximal concentration of 100 nmol/L) and PMA (500 nmol/L) also enhanced \([\text{H}]\)DOG uptake by 25.3% and 39.3%, respectively. The combination of ET (100 nmol/L) plus insulin (500 nmol/L) or PMA plus insulin did not exert a further effect on \([\text{H}]\)DOG uptake compared with insulin alone. Additional experiments were undertaken using lower concentrations of insulin and ET (Fig 3B). These experiments were performed in cells obtained from different individuals than those described in Fig 3A. ET (10 nmol/L) and insulin (50 nmol/L) enhanced \([\text{H}]\)DOG uptake by 18.0% and 35.0%, respectively. The combined effect of both agents at these concentrations was not additive (37.5% above control), suggesting that insulin and ET act through similar mechanisms.

The effect of 50 nmol/L calphostin C on the \([\text{H}]\)DOG uptake (not shown) was examined in further experiments. Although at this concentration the insulin- and ET-induced increases in \([\text{H}]\)DOG uptake were totally ablated by calphostin C, this agent also exerted an inhibitory effect on basal \([\text{H}]\)DOG uptake (a decline of...
FIG 3. Bar graphs show [3H]deoxy-o-glucose ([3H]DOG, shown as DOG in figure) uptake in cultured human skeletal myoblasts after treatment with maximal (A) and submaximal (B) doses of insulin and endothelin. For A, C indicates basal uptake; ET, preincubation for 60 minutes with 100 nmol/L endothelin; INS, preincubation for 60 minutes with 500 nmol/L insulin; ET+INS, preincubation for 60 minutes with both ET and INS; PMA, preincubation for 5 minutes with 500 nmol/L phorbol 12-myristate 13-acetate; and PMA+INS, preincubation for 60 minutes with INS and for 5 minutes with PMA. For B, C indicates basal uptake; ET, preincubation for 60 minutes with 10 nmol/L ET; INS, preincubation for 60 minutes with 50 nmol/L insulin; and ET+INS, preincubation for 60 minutes with both ET and INS. For A, * denotes significance at \( P = 0.001 \) for C vs INS, \( P < 0.0012 \) for C vs ET, and \( P = 0.001 \) for C vs PMA. For B, * denotes significance at \( P = 0.0023 \) for C vs ET and \( P = 0.0001 \) for C vs INS and for C vs ET+INS. Values in A and B represent mean ± SEM from experiments using SKMBs from five subjects. Different subjects were used for experiments in A and B.

24.8%). Moreover, at higher concentrations, calphostin C exerted a further inhibition on the basal [3H]DOG uptake. Thus, the effect of calphostin C on insulin- and ET-mediated glucose transport appears to be nonspecific, since calphostin C also inhibits the basal glucose transport. It is possible that 24-hour exposure to a high glucose medium in preparation for the [3H]DOG uptake experiments stimulated PKC, and as a result, the basal [3H]DOG uptake also was partially PKC dependent. Alternatively, maintaining the cells for 24 hours in 0.1% FBS also could result in slight activation of PKC. There was no apparent effect of calphostin C on cellular viability (measured by trypan blue exclusion).

In further experiments we examined the effect of Ang II on the Ca\(^{2+}\) profile and [3H]DOG uptake in SKMBs. Ang II, like ET, produced robust Ca\(^{2+}\) responses in 1.8 mmol/L Ca\(^{2+}\) HBS and Ca\(^{2+}\)-free HBS that were not modified by Ca\(^{2+}\) channel blockers (Fig 4). However, unlike ET, Ang II had no apparent effect on [3H]DOG uptake in these cells (not shown).

L6 Cells

Cultured human skeletal muscle cells are not available for routine experimentation. It is difficult to obtain differentiated myotubes from human skeletal muscle on a large scale and from different subjects. Therefore, we
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also examined the effect of ET on differentiated myotubes from L6 cells. As in human SKMBs, ET induced Ca transients and enhanced [3H]DOG uptake in these cells in a concentration-dependent manner (Fig 5). Basal [3H]DOG uptake in L6 myotubes was 34.2 ±0.5 pmol/mg protein per minute (from 14 preparations). As with human SKMBs, the stimulatory effect of ET on [3H]DOG uptake was not additive with that of insulin (Fig 5). Insulin alone at concentrations of 25, 100, and 1000 nmol/L, respectively, increased [3H]DOG uptake by 38.6%, 82.0%, and 72.1% above basal level. Combinations of 25 nmol/L insulin and 10 nmol/L ET, 100 nmol/L insulin and 50 nmol/L ET, and 1000 nmol/L insulin and 100 nmol/L ET increased [3H]DOG uptake by 40.2%, 61.7%, and 87.0%, respectively.

Further experiments in L6 myotubes demonstrated two classes of ET receptors on these cells with similar density as SKMBs (=15,000 per cell). More than 90% of these receptors were of the ETA type, and the remaining were of the ETB type (Z.F., X.Y.Y., A.A., unpublished data).

**Discussion**

The presence of specific ET receptors on cultured human SKMBs and their robust ET-evoked Ca response indicate that the effect of ET on SKMBs is receptor mediated. Furthermore, the ET- and PMA-induced increases in [3H]DOG uptake and the inhibition of this process by calphostin C suggest that the glucose transport in human SKMBs is mediated partially through activation of PKC. Perhaps as important is the observation that the actions of ET and insulin on [3H]DOG uptake are not additive. Thus, conditions associated with increased levels of ET and similar vasoactive agents acting through PKC in the vicinity of skeletal muscle may modify the response of this tissue to insulin. By the same token, elevated levels of insulin and glucose also may modify glucose transport resulting from exposure to vasoactive agents via Ca-dependent and PKC-independent mechanisms. At present, the mechanisms responsible for the ET-evoked increase in glucose uptake are unknown, but they may relate to enhanced glucose utilization distal to the transport across the plasma membrane. Additionally, the confirmation of the effect of ET in L6 myotubes indicates that the effect of this agent is exerted not only on dedifferentiated but also on differentiated skeletal muscle cells.

Elevated extracellular glucose can stimulate PKC. This stimulation not only modulates the expression of insulin receptors but also modifies the density of receptors to vasoactive agents such as Ang II. In turn, vasoactive agents can directly alter glucose metabolism in some tissues. As important, PKC participates in ET gene expression in endothelial cells. Collectively, these observations suggest reciprocal interactions between vasoactive factors and pathways controlling the glucose metabolic status at the cellular level. PKC appears to play a central role in this relation.

It is rather unlikely that factors of endothelial origin, which act through the Ca, and PKC pathways, would reach sufficiently high concentrations in the vicinity of skeletal muscle to trigger muscle contraction through a rise in Ca. Such a scenario would result in nonvoluntary muscular activity. The question therefore arises whether these agents can alter glucose metabolism without reaching the Ca, threshold that is necessary to initiate skeletal muscle contraction. The answer to this question is in the affirmative, as a recent work by Youn et al has demonstrated that raising the Ca, in the epitrochlearis muscle of the rat to a level too low to cause contraction was sufficient to evoke a substantial increase in glucose transport. It is very unlikely, however, that the ET-evoked rise in Ca, is the direct signal for enhanced [3H]DOG transport, as this rise was only transient and the posttransient Ca, was barely above the resting Ca, concentration. Moreover, Ang II also can evoke a Ca, transient in human SKMBs, but it does not exert any effect on [3H]DOG uptake in these cells. Thus, mechanisms in addition to a rise in Ca, must be stimulated by ET to evoke changes in skeletal muscle cell glucose transport.

By releasing vasoactive agents and other factors, the endothelium serves not only as a barrier, separating VSMCs from circulating cells and other blood elements, but also as an active and dynamic participant in vascular functions. The present study suggests that the endothelium also may participate in skeletal muscle glucose metabolism. The signals for the release of endothelial factors originate from both the luminal and counterluminal aspects of the endothelium; they include physical factors, eg, stretch and shear forces, circulating factors, as well as paracrine and autocrine elements within the vascular wall. The final status of a particular vascular bed and skeletal muscles supplied by this bed may therefore depend on the combined effect of factors acting on or originating from the endothelium. For
instance, the expression of mRNA for ET, the release of ET, or both are regulated by Ang II, thrombin, vasopressin, low-density lipoproteins, and nitric oxide. The role of Ang II is of particular interest in this regard, as the effect of Ang II may reflect its circulating levels or local production in the vascular wall. Here a reciprocal interaction exists between Ang II and ET. Ang II modifies the expression of ET, but Ang II also is regulated by ET through the stimulatory effect of ET on angiotensin-converting enzyme (ACE). Several observations support the idea that the special relation between ET and Ang II plays an important function in the pathophysiology of essential hypertension. First, the ACE inhibitor captopril prevents chronic hypertension produced by the systemic administration of ET in the rat; second, captopril improves the impaired endothelium-dependent vasodilation in patients with essential hypertension; and third, Ang II stimulates ET production and augments the contractility of resistance vessels of the spontaneously hypertensive rat to a greater extent than the Wistar-Kyoto rat. Taken together, these observations suggest that at least some forms of essential hypertension are marked by hyperactivity of ET and Ang II or increased sensitivity to these agents of the peripheral circulation. Ang II redistributes blood to skeletal muscle and it also directly enhances glucose uptake in VSMCs. However, Ang II exerts no effect on glucose uptake in human SKMBs. Thus, the observations that ACE inhibitors improve insulin resistance in essential hypertension and in non–insulin-dependent diabetes mellitus complicated by hypertension (reviewed in Reference 48) cannot be explained by blunting a direct effect of Ang II on glucose metabolism in human skeletal muscle. However, ACE inhibitors would be expected to decrease the expression of ET in the peripheral circulation through inhibiting Ang II formation. Such a process may thus indirectly modify glucose metabolism in skeletal muscle. Viewed from this prospective, insulin resistance in essential hypertension could result from enhanced glucose utilization by skeletal muscle due to increased levels of vasoactive agents like ET. This is a reasonable assumption, provided that the cellular pathways for the enhancement of glucose uptake by some vasoactive agents are mediated through mechanisms similar to those of insulin. Such resistance to insulin would be attenuated by eliminating the direct effect of vasoactive agents on glucose metabolism in skeletal muscle. In summary, the present study demonstrates that ET enhances glucose uptake in cultured human SKMBs and L6 myotubes. It is likely that some other vasoactive agents can exert the same effect. Thus, an imbalance between vasoactive agents and vasodilators in vascular beds of skeletal muscles may not only increase the PVR and promote hypertension but also alter glucose metabolism in skeletal muscle.

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