β2-Adrenergic receptor stimulation in vivo induces apoptosis in the rat heart and soleus muscle

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Burniston, Jatin G., Lip-Bun Tan, and David F. Goldspink. β2-Adrenergic receptor stimulation in vivo induces apoptosis in the rat heart and soleus muscle. J Appl Physiol 98: 1379–1386, 2005.—High doses of the β2-adrenergic receptor (AR) agonist clenbuterol can induce necrotic myocardial cell death in the heart and slow-twitch skeletal muscle of the rat. However, it is not known whether this agent can also induce myocyte apoptosis and whether this would occur at a lower dose than previously reported for myocyte necrosis. Male Wistar rats were given single subcutaneous injections of clenbuterol. Immunohistochemistry was used to detect myocyte-specific apoptosis (detected on cryosections via a caspase 3 antibody and confirmed with annexin V, single-strand DNA labeling, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling). Myocyte apoptosis was first detected at 2 h and peaked at 4 h after clenbuterol administration. The lowest dose of clenbuterol to induce cardiomyocyte apoptosis was 1 μg/kg, with peak apoptosis (0.35 ± 0.05% P < 0.05) occurring in response to 5 mg/kg. In the soleus, peak apoptosis (5.8 ± 2%; P < 0.05) was induced by the lower dose of 10 μg/kg. Cardiomyocyte apoptosis was detected throughout the ventricles, atria, and papillary muscles. However, this damage was most abundant in the left ventricular subendocardium at a point 1.6 mm, that is, approximately one-quarter of the way, from the apex toward the base. β-AR antagonism (involving propranolol, bisoprolol, or ICI 118551) or reserpine was used to show that clenbuterol-induced myocardial apoptosis was mediated through neuromodulation of the sympathetic system and the cardiomyocyte β1-AR, whereas in the soleus direct stimulation of the myocyte β2-AR was involved. These data show that, when administered in vivo, β2-AR stimulation by clenbuterol is detrimental to cardiac and skeletal muscles even at low doses, by inducing apoptosis through β1- and β2-AR, respectively.

clenbuterol; caspase 3; skeletal muscle; myocardium; adrenergic receptor agonists/antagonists

THE β2-ADRENERGIC RECEPTOR (AR) agonist clenbuterol can induce hypertrophy of striated muscles and may also lead to the development of a faster contracting muscle phenotype (34). Because of this, clenbuterol is used as an adjunct to ventricular assist devices (22) and has also been proposed as a possible anticachectic agent (2). Chronic heart failure is also associated with cachexia, and a reduction in skeletal muscle mass is indicative of a poorer prognosis for such patients (1). Hence, in addition to its specific use as an adjunct to ventricular assist devices, the trophic effects of this β2-AR agonist may also benefit patients with chronic heart failure by counteracting the associated loss of muscle mass. For similar reasons, competitive and recreational athletes and body builders have long used (or abused) clenbuterol in their search for enhanced performance (8). The illicit use of this agent persists to the present day (11). However, administration of clenbuterol does not necessarily lead to improved muscle performance (5, 13, 24, 26, 28, 35). Case reports include body builders who present with tachycardia (6), myocardial infarction (19), and end-stage renal disease (21). It is therefore important to ascertain whether clenbuterol can induce apoptosis in striated muscles, similar to the well-known toxic effects of catecholamines (17, 31).

Many of the animal studies that have investigated the anabolic effects of clenbuterol have used very high doses, typically in the region of 1–5 mg·kg−1·day−1. Work from our laboratory has shown (4) that doses as little as 100 μg/kg can induce cardiomyocyte necrosis. These data support those of Duncan et al. (13), who observed an increase in the collagen content of the hearts of rats administered clenbuterol and a high incidence of sudden cardiac death when clenbuterol administration was combined with endurance exercise. Apoptotic cell death can often be induced by lesser stimuli than required to induce necrosis (23). Whether the doses of clenbuterol administered differentiate between its well-known anabolic effects (34) and the more recently reported injurious effects (4, 13, 26, 39) is unknown. But if it does, a rigorously investigated dose dependency is required to find the highest dose of clenbuterol that does not induce myocyte death and could therefore potentially be used as a therapeutic intervention for muscle wasting.

Much is already known about the intracellular mechanisms involved in β2-AR stimulation (3, 38, 46, 47, 49, 50); available information suggests that β2-AR stimulation is antiapoptotic and that it may even be beneficial to the failing heart. These suggestions are based on data from studies conducted on isolated cardiomyocytes in vitro (7, 45), β2-AR knockout studies (9, 33) and short-term evaluation of transgenic overexpression models (16, 29). However, detrimental effects have also been reported in studies that have overexpressed β2-AR (10, 27). Therefore, before the potential benefits of β2-agonists can be extrapolated into the therapeutic arena, it is essential to know whether other mechanisms, apart from the known subcellular ones, are also involved. Studies on isolated cells in vitro and genetically manipulated models can provide detailed insight into intracellular mechanisms, but the applicability of information derived from these systems often needs to be verified by investigations made on intact wild-type animals. The present work tests the hypothesis that, when administered in vivo, clenbuterol will induce apoptosis in the heart and skeletal muscles and that this damage will occur at doses currently assumed to be safe.
METHODS

Animal care and tissue harvesting. All experimental procedures were conducted under the British Home Office Animals (Scientific Procedures) Act 1986 and were approved by the local ethical review committee. Male Wistar rats (289 ± 19 g) were bred in-house in a conventional colony, housed in controlled conditions of 20°C, 45% relative humidity, and a 12-h light (0600–1800) and 12-h dark cycle, with water and food available ad libitum.

β-Agonists (clenbuterol and isoproterenol) and antagonists (propranolol, bisoprolol, and ICI 118551) were administered by subcutaneous (sc) injection. The time course (0–24 h) of clenbuterol-induced myocyte apoptosis was investigated, and these data were used to optimize the experimental variables for investigating the dose dependency of myocyte death over the range 1 ng to 5 mg/kg clenbuterol. The topographical distribution of cardiomyocyte apoptosis was investigated along the longitudinal axis of the heart in response to the peak-damaging dose of clenbuterol. By using an optimized model of clenbuterol-induced apoptosis, selective β-AR antagonists and the synaptic vesicle transport blocker reserpine were used to investigate the β-AR subtype that mediated the clenbuterol-induced myocyte apoptosis. This was confirmed further by comparison with the non-selective β-agonist isoproterenol.

After the respective experimental procedures, rats were rendered unconscious and killed by cervical dislocation. The heart and soleus muscles were quickly isolated. The atria were dissected and mounted separately with a piece of liver as a support. The remaining great vessels were removed and the ventricles were mounted apex uppermost. A segment of the midbelly of each soleus was mounted in transverse section and supported with liver. Tissues were snap frozen in supercooled isopentane and stored at −80°C, before cryosectioning (5 μm thick).

Immunohistochemical detection of apoptosis. Routine detection of apoptosis was achieved by using an anti-caspase 3 antibody (Ab; R&D Systems, Minneapolis, MN). Heat-denatured (3 min at 96°C) anti-caspase 3 Ab was used as the negative control, with all other stages being identical. In addition, all experiments included a group of control animals. These received the saline vehicle only, to detect the presence of any background apoptosis induced by either experimental stress or tissue processing.

To confirm apoptosis labeling using the caspase 3 Ab, a subset of animals (n = 3, in each group) was administered annexin V-biotin (Nexins Research, Kattendijke, The Netherlands) to detect the externalization of phosphatidylserine in vivo. Annexin V-biotin (25 mg/kg) was administered intravenously 3 h 15 min after the administration of 5 mg/kg of either isoproterenol, clenbuterol (experimental groups), or saline vehicle only (control group). Muscles were harvested at the optimized time point (4 h after β-agonist administration) and processed by standard immunohistochemical techniques, as described previously (12).

Formamide-induced DNA denaturation, detected by using an anti-single strand DNA (ssDNA) Ab, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) were used in vitro to confirm the nuclear changes concomitant with apoptosis. Cryosections of muscles taken from control and experimental animals were heated in formamide as described by Frankfurt and Krishan (15). The denatured DNA of apoptotic cells was then detected by using an anti-ssDNA Ab (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Strand breaks in the DNA of apoptotic cells were detected by using a commercially available TUNEL kit (R&D Systems) according to the instructions provided.

Microscopy and image analysis. Cryosections stained with caspase 3 were used to quantify the incidence of clenbuterol-induced myocyte death in the heart and soleus muscles. With the exception of the topographical investigation, cardiomyocyte death in the heart was only quantified in the subendocardial region of the left ventricle (LV). For each section, six to eight fields of view (×100 magnification), encompassing the entire subendocardial region (~10^4 cells), were digitized. Positive staining (apoptosis) was differentiated from the hematoxylin background and quantified by image analysis, and the incidence of myocyte death was expressed as percent area relative to each field of view. The coefficient of variation with this technique was 4.7%.

To quantify myocyte death in the heart, three random fields of view (×100 magnification) across each transverse section were digitized. Both injured and viable fibers were counted (>700 fibers), and the number of damaged fibers was expressed as a percentage of the total. The coefficient of variation for this technique was 3.5%.

Statistical analyses. All data are presented as means ± SE. Data were analyzed by one-way analysis of variance with multiple post hoc analyses. P values of <0.05 were used to indicate statistical significance.

RESULTS

Administration of the saline vehicle alone did not induce any apoptosis in the heart (i.e., zero baseline in the control group), whereas caspase 3-positive cells were found in both striated muscles after the administration of either isoproterenol or clenbuterol (Fig. 1). The specificity of the caspase 3 immunoperoxidase technique for detecting apoptosis was confirmed by annexin V-biotin, administered in vivo, and the ssDNA Ab and TUNEL techniques in vitro (Fig. 1).

The time course of apoptotic cell death was similar for both striated muscles. The peak incidence of apoptosis was measured 4 h after clenbuterol administration (Fig. 2). This optimum time point was used in all subsequent experiments.

Cardiomyocyte apoptosis was only observed in animals that had received doses of 1 μg/kg clenbuterol or higher (Fig. 3A). In the soleus, a very low incidence of apoptosis was observed in some control muscles taken from animals that had been administered saline only. The incidence averaged only 0.1 ± 0.06% of fibers across the whole group. The administration of 10 μg/kg clenbuterol induced significantly (P < 0.05) more apoptosis than that seen in the saline controls (Fig. 3B). Doses of clenbuterol above 10 μg/kg did not further increase the amount of myocyte apoptosis in the soleus.

In the heart, clenbuterol-induced apoptosis was evident along the entire longitudinal axis (0–9.5 mm) of the LV subendocardium. Peak apoptosis occurred 1.6 mm from the apex (Fig. 4). When investigated in more detail, i.e., across the entire transverse section at this peak (1.6 mm), apoptosis in the LV subendocardium (0.35 ± 0.05%) was approximately four times greater (P < 0.05) than that in the right ventricular subendocardium (0.09 ± 0.01%) or right or left subepicardium (0.08 ± 0.01%).

As in the ventricles, no baseline apoptosis was found in the papillary muscles or atria of control hearts harvested from animals that had received only the saline vehicle. However, after clenbuterol treatment, the incidence of apoptosis in random samples of the papillary muscles was 0.14 ± 0.02% and 0.02 ± 0.01% in the atria.

Prior β2-AR selective blockade by means of ICI 118551 significantly (P < 0.05) reduced (by 80%) the incidence of the clenbuterol-induced apoptosis in the heart (Fig. 5A). Propranolol, providing both β2- and β1-AR blockade, was similarly (98%) effective. Prior β1-AR selective blockade (bisoprolol) also provided significant (P < 0.05) protection (98%) against the clenbuterol-induced (β2-AR agonist) apoptosis. Because clenbuterol may facilitate additional norepinephrine (NE) re-
lease from the sympathetic nerve terminals by stimulating presynaptic β2-AR, reserpine was administered to deplete the NE-releasing capacity of the sympathetic system. The prior administration of reserpine significantly (P < 0.05) decreased (68%) the clenbuterol-induced cardiomyocyte apoptosis.

In the solei of the same animals, only prior β2-AR blockade using either ICI 118551 or propranolol was effective (93% and 88%, respectively) in protecting against clenbuterol-induced apoptosis. Prior intervention with bisoprolol or reserpine did not reduce the incidence of clenbuterol-induced apoptosis in this skeletal muscle (Fig. 5B).

The protective effects of prior administration of different doses of the β2-AR selective antagonist, ICI 118551, were investigated against peak damaging doses of either clenbuterol (a β2-AR selective agonist) or isoproterenol (a β1- and β2-agonist). In the heart, the prior administration of 10 mg/kg ICI 118551 significantly (P < 0.05) reduced (by 72%) the incidence of apoptosis in response to clenbuterol (Fig. 6A), whereas the incidence of isoproterenol-induced apoptosis was not significantly affected (22% reduction). In the soleus, the effect of prior β2-AR blockade was the same against either clenbuterol or isoproterenol, with doses of 1 mg/kg ICI 118551.

Fig. 1. β-Adrenergic receptor (AR) agonist-induced apoptosis in the left ventricular (LV) subendocardium and soleus muscle. Caspase 3 staining of cryosections (×100 magnification) of control heart (A) or soleus (C) after the administration of the saline vehicle only. Caspase 3-positive myocytes (brown labeling) in the heart and the soleus (B and D, respectively; ×100 magnification) of animals administered 5 mg/kg clenbuterol. Images at higher magnification (×1,000) did not show the formation of apoptotic bodies in either skeletal (E) or cardiac (I) myocytes that stained positive for caspase 3. Annexin V-biotin-labeled cardiomyocytes (arrows) were also detected after the administration of either isoproterenol (F; ×100 magnification) or clenbuterol (H; ×200 magnification). When serial cryosections of hearts from clenbuterol-treated animals were stained for annexin V-biotin (G) and caspase 3 (H; ×200 magnification), colocalization of the two markers within individual cardiomyocytes was observed. These cryosections of the heart (×1,000 magnification) also had positive brown staining (arrows) when exposed to formamide and the single-strand DNA antibody (J) or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (K), confirming apoptosis by 4 different methods.
or greater providing statistically significant \( P < 0.05 \) protection against \( \beta \)-agonist-induced apoptosis (Fig. 6B).

**DISCUSSION**

Administration in vivo of the \( \beta_2 \)-AR selective agonist clenbuterol leads to the activation of caspase 3, the externalization of phosphatidylserine, and increases the sensitivity of myocyte DNA to heat denaturation and strand breaks (Fig. 1), each of which is a hallmark of apoptosis (12, 15, 37). This is the first time that myocyte death by the apoptotic pathway has been described in response to this muscle growth-promoting agent. In contrast, the cardiotoxic effects of the nonselective \( \beta \)-agonist isoproterenol are well established (31). So in addition to the routine immunohistochemical controls, isoproterenol was used as a positive control to show that annexin V-biotin also detected the myocyte death in vivo (Fig. 1). These observations are in keeping with our previous findings that catecholamines, either natural (epinephrine and NE) or synthetic (isoprotere-

The greater sensitivity of the apical region of the heart to clenbuterol (Fig. 4), and other \( \beta \)-AR agonists (31), may be related to the observed regional differences in cardiac uptake activity and the abundance of \( \beta \)-AR kinase-1 (43). These data suggest that random sampling from the heart may give rise to misleading interpretations, and more investigations need to be undertaken in vivo to fully understand this phenomenon.

Only the highest dose of 5 mg/kg clenbuterol induced a statistically significant \( (P < 0.05) \) amount of apoptosis 0.35 \( \pm \) 0.09\% (means \( \pm \) SE) of the area of the LV subendocardium (Fig. 3A); this is equivalent to 40–50 cardiomyocytes from a sample of \( \sim 10^4 \) cells. However, the threshold dose for induc-

![Fig. 2. Time course of clenbuterol-induced apoptosis. Independent groups of animals received single subcutaneous (sc) injections of 5 mg/kg clenbuterol (experimental rats; \( n = 4–6 \), in each group) or saline only (control rats; \( n = 3 \)). Groups of animals were killed at specific time points from 0 (controls) to 24 h after the administration of clenbuterol. Myocyte apoptosis, detected by using caspase 3, was quantified in the LV subendocardium (A) and soleus muscle (B). Data are presented as means \( \pm \) SE. *\( P < 0.05 \) significantly different from 0-h saline control group.

![Fig. 3. Dose dependency of clenbuterol-induced myocyte death. Clenbuterol was administered sc to 8 independent groups (\( n = 6–8 \), in each group) of rats over the range 1 ng to 5 mg/kg. The control group (\( n = 4 \)) received saline only. All animals were killed 4 h (time of peak incidence of apoptosis derived from Fig. 2) after the administration of clenbuterol. Myocyte apoptosis, detected using caspase 3, was quantified in the LV subendocardium (A) and soleus muscle (B). Data are presented as means \( \pm \) SE. *\( P < 0.05 \) significantly different from saline control group. In the heart, any myocyte death above that of the zero baseline must be biologically significant, although not necessarily considered statistically different from the saline controls.](http://jap.physiology.org/content/98/4/1382)
ing cardiomyocyte apoptosis was 1 µg/kg clenbuterol. Because no apoptosis was detected in the hearts of control animals (administered saline only), any myocyte death induced by the administration of clenbuterol must be physiologically significant and, if repeated and cumulative over time, will affect the function of the heart. Recently, an incidence rate of only 23 TUNEL-positive cardiomyocytes per 10^6 viable cells (i.e., comparable to the damage induced here in response to 10 µg/kg clenbuterol) was shown to be sufficient to induce lethal dilated cardiomyopathy in mice over a period of 8 wk (44). The cell death reported here in response to single doses of clenbuterol might, therefore, explain the previously reported myocardial fibrosis and sudden cardiac death in rats after chronic administration of high doses of clenbuterol (13).

Clenbuterol has recently been used as an adjunct to the implantation of LV assist devices (The Harefield Protocol) as a bridge to recovery and has been shown to aid the reverse remodeling of the myocardium (22, 48). These patients also receive “combination therapy” that includes β1-AR blockade. It is likely, therefore, that in this case the heart will be protected from the myotoxic effects of clenbuterol, as explained below. However, their skeletal musculature will remain vulnerable to β2-AR-induced myocyte death. The potential additional loss of skeletal muscle bulk in already severely ill patients, together with the “knock-on” effects on their protein metabolism and exercise capacity, warrants further investigation before the use of clenbuterol becomes widely accepted as a standard therapeutic intervention. With regard to the illicit use of clenbuterol, the philosophy of “the more you take, the greater the benefit” must engender a cause for concern.

Studies investigating the effects of β1- or β2-AR signaling on adult rat ventricular myocytes in vitro (7, 49) suggest that β1-AR stimulation is proapoptotic whereas β2-AR stimulation is antiapoptotic. Accordingly, overexpression of cardiac β1-AR is detrimental (14) and so too is genetic removal of β2-AR (33). However, β-agonists such as clenbuterol can accumulate in high concentrations in the heart (41), and in the present study administration of this β2-AR selective agonist in vivo induced apoptosis in the rat myocardium. β2-AR selective antagonism significantly suppressed, and combined β1- and β2-AR antagonism almost completely prevented clenbuterol-induced apoptosis (Fig. 5A), suggesting β2-AR involvement. In
innervation of skeletal muscle is markedly less than in the heart (32) and is only associated with the muscle vasculature (20). Furthermore, ~90% of the β-AR in skeletal muscle are of the β2 subtype (i.e., have a greater affinity for epinephrine than NE) and are associated predominantly with the slow-twitch fibers (25); the remaining 10% being β1-AR associated with the vasculature. Hence, whereas an enhanced release of NE, as described above, could act via the myocardial β1-AR, the same mechanism is most unlikely to apply to skeletal muscle fibers. It is therefore perhaps not surprising that fiber damage in skeletal muscle is mediated only by the β2-AR (4, 31, 42). This was further confirmed in the present work (Fig. 6B) using the peak dose of 10 μg/kg clenbuterol. However, these data do suggest that a fundamental difference may exist between the β2-AR of skeletal muscle and those found in the heart. The β2-AR of isolated cardiomyocytes are known to signal through both G protein αi and G protein αo pathways, with G protein αo signaling being predominant (47). Clearly, the skeletal muscle β2-AR appears to be functionally more similar to the cardiac β1- than β2-AR, because it too stimulates cell death. This suggests that the skeletal muscle β2-AR may signal predominantly through the stimulatory G protein αi (similar to the cardiac β1-AR) rather than G protein αo pathway. Unlike the cardiac β2-AR, there is currently very little information available concerning the interaction of the skeletal muscle β2-AR with G proteins to either support or reject this possible explanation. So, although the observed difference in receptor mediation of myocyte death may be linked with the relative predominance of β2-AR in skeletal muscle and β1-AR in the myocardium, possible mechanistic differences in the β2-AR G protein interactions in these two striped muscles remain to be explored.

Taking all our experimental observations together, the most likely mechanism for clenbuterol-induced toxicity on cardiac myocytes appears to be an injurious effect, not directly via the cardiomyocyte β2-AR, but indirectly via stimulation of the β2-AR of presynaptic nerve terminals, which consequently augments the release of NE (30). This observation that β2-AR activation in vivo can induce apoptosis does not negate the putative antiapoptotic effects of β2-AR stimulation in vitro (7, 46, 47, 49) but instead shows that the antiapoptotic effect of β2-AR stimulation is relatively small and easily overwhelmed by the concomitant indirect β1-AR stimulation that occurs in vivo. Similarly, the assumed protective role of β2-AR (33) is based on data from wild-type and β2-AR knockout mice administered 120 μg·g−1·day−1 isoproterenol. After studying the dose dependency, we found that the peak damaging dose for isoproterenol in rats is 5 mg/kg (31). Because of the high dose used in the study by Patterson et al. (33), any additional stimulation of the cardiomyocyte β1-AR via isoproterenol’s stimulation (β2-AR) of presynaptic vesicles (augmenting their release of NE) would be inconsequential. Therefore, the influence of neuromodulation of the sympathetic system in their experiment is effectively negated and so the only effect measured is that at the cardiomyocyte β2-AR (i.e., antiapoptotic).

These data confirm that the administration of a β2-AR selective agonist to the whole animal directly induces apoptosis in skeletal muscle and indirectly induces cardiomyocyte apoptosis via its sympathoexcitatory effect. These findings provide a wider perspective on the use of β2-agonists and may explain the adverse cardiovascular effects seen in chronic obstructive

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**Fig. 6. Dose-dependent effects of β2-AR selective antagonism on clenbuterol- or isoproterenol-induced apoptosis.** Rats were randomized into 10 independent groups (n = 4 in each group) and were given single injections sc of either saline only (control group) or ICI 118551 (β2-AR antagonist) over the range 100 μg to 10 mg/kg. One hour after β-antagonist administration, the independent groups of animals were challenged (sc) with peak-damaging doses of either 10 μg or 5 mg/kg clenbuterol (to induce peak apoptosis in the soleus or heart, respectively), or 5 mg/kg isoproterenol. Animals were killed 4 h (peak time) after the administration of clenbuterol and the incidence of apoptosis quantified in the LV subendocardium and soleus muscle. The degree of protection afforded to the LV subendocardium (A) and soleus muscle (B) against β-agonist-induced apoptosis was calculated. The value of 100% represents the incidence of apoptosis induced by a peak-damaging dose of either clenbuterol (●) or isoproterenol (○), unopposed by β2-AR inhibition. In the heart, administration of 10 mg/kg ICI 118551 provided significant (P < 0.05) protection against clenbuterol, but not isoproterenol. In the soleus, doses of 1 mg/kg ICI 118551 or above afforded significant (P < 0.05) protection against both clenbuterol- and isoproterenol-induced damage.

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**Table 1.**

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<th>Condition</th>
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**Note:** Dose-dependent effects of β2-AR selective antagonism on clenbuterol- or isoproterenol-induced apoptosis.
pulmonary disease patients receiving β2-agonist therapy (36). Accordingly, β2-agonist administration could only be used as a therapy if combined with β1-AR antagonism to protect the heart, but this would need to be weighed against the direct adverse effects on the skeletal musculature.

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