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Peroxisome proliferator activated-receptor agonism and left ventricular remodeling in mice with chronic myocardial infarction

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1 Peroxisome proliferator activated receptor γ (PPARγ) has been implicated in several cellular pathways assumed to beneficially affect heart failure progression. In contrast, population-based studies demonstrate an increased incidence of heart failure in patients treated with PPARγ agonists. Therefore, we examined the effect of pioglitazone, a PPARγ agonist, on chronic left ventricular remodeling after experimental myocardial infarction (MI) in mice.

2 Mice were treated with placebo or pioglitazone (20 mg kg^{-1} by gavage) from week 1 to week 6 after ligation of the left anterior descending artery. Serial transthoracic echocardiography was performed at weeks 1, 3, and 6.

3 Over 6 weeks, there was no difference in mortality (placebo 12%, pioglitazone 10%). Echocardiography showed significant left ventricular dilatation in animals with MI (week 6, end-systolic area, placebo sham 9.6 ± 1.3 vs placebo MI 14.4 ± 2.5 mm^2). However, there was no difference between the placebo and pioglitazone groups (week 6, end-systolic area, pioglitazone MI 14.8 ± 2.9 mm^2, $P = $ NS vs placebo).

4 Moreover, there were no changes in metabolic parameters, inflammation, and collagen deposition. Endothelial function in the aorta was not changed by PPARγ activation.

5 In conclusion, PPARγ activation did not adversely affect left ventricular remodeling and survival in mice with chronic MI. However, we were also not able to identify a protective effect of pioglitazone.
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Abbreviations: 2D, two-dimensional; ACh, acetylcholine; CHF, chronic heart failure; EDA, end-diastolic area; EDD, end-diastolic diameter; ESD, end-systolic diameter; ESA, end-systolic area; FS, fractional shortening; IL, interleukin; IVS, interventricular septum; LV, left ventricle; MI, myocardial infarction; PPAR, peroxisome proliferator activated receptor; PW, posterior wall; SNP, sodium nitroprusside; TNF, tumor necrosis factor; TZD, thiazolidinedione; wt, weight

Introduction

Peroxisome proliferator activated receptor γ (PPARγ) belongs to a family of three hormone receptors: PPARα, PPARδ, und PPARγ (Marx & Hombach, 2001). It is a member of the nuclear receptor superfamily of ligand activated transcription factors. For activation, PPARγ forms heterodimers with another nuclear receptor such as retinoid X receptor. The resulting complex interacts with PPAR response elements and alters the transcription of numerous target genes. PPARγ is most abundantly expressed in adipose tissue, the intestine, and cells of the immune system. However, it is also expressed at a substantially lower level in the heart (Takano *et al*., 2000) and in skeletal muscle. In adipose tissue, PPARγ stimulates transcription of genes involved in lipid metabolism and promotes adipocyte differentiation. In inflammatory cells and vascular smooth muscle cells, PPARγ-dependent signaling suppresses the production of proinflammatory cytokines (Jiang *et al*., 1998) and inhibits proliferation and migration (Marx *et al*., 1998). PPARγ can be activated by natural ligands, including prostaglandin D_2 derivates, linoleic acid, and components of oxidized LDL, as well as by synthetic antidiabetic drugs, the so-called glitazones or thiazolidinediones (TZD) such as rosiglitazone and pioglitazone. TZDs are in clinical use as antidiabetics. In adipocytes, TZDs increase insulin responsiveness and their capacity of glucose uptake. Furthermore, TZDs increase glucose disposal in the skeletal muscle and reduce glucose output of the liver (Schoonjans & Auwerx, 2000).

The function of PPARγ and TZDs in the heart and their roles in cardiovascular diseases such as chronic heart failure (CHF) is far less defined (Bishop-Bailey, 2000). In experimental studies, TZDs reduce the production of endothelin (Buchanan *et al*., 1995) and proinflammatory cytokines (Sidhu & Kaski, 2001). Since these mediators are involved in the progression of heart failure, a protective effect of TZDs may be postulated. In contrast, a retrospective population-based cohort analysis evaluating the relationship between glitazone treatment and heart failure risk in type II diabetics demonstrated a significant increase in heart failure development by TZDs independently of age and other risk factors (Delea *et al*.,

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2002). To resolve this paradox, we evaluated the influence of the TZD pioglitazone on left ventricular remodeling after experimental myocardial infarction (MI) in mice. However, we did not detect any differences between the groups with regard to left ventricular dilatation, mortality, and endothelial function.

Methods

Animals and surgery

Male C57Bl6 mice 8–12 weeks old with a body weight of 20–28 g underwent left coronary artery ligation to induce MI, as described before (Frantz *et al*., 1999). At day 7, animals were randomized to receive either placebo or pioglitazone at a standard dose of 20 mg kg^{-1} suspended in 0.5% methylcellulose by gavage once a day (Simoncikova *et al*., 2002). The dose of 20 mg kg^{-1} was chosen since it has full glucose-lowering activity in diabetic mice (Ikeda *et al*., 1990). Therefore, the dose of 20 mg kg^{-1} in mice is equivalent with regard to glucose-lowering activity to the dose used in humans. The Standing Committee on Animal Research from our institution approved the animal study protocol.

Imaging procedure

Echocardiographic studies were performed under light anesthesia with spontaneous respiration using intraperitoneal tribromoethanol/amylene hydrate (Avertin) 2.5% w v^{-1} solution (8 μl g^{-1} bodyweight) as recently described (Ducharme *et al*., 2000). Avertin was chosen due to its negligible hemodynamic effects at this dose. The imaging was performed by an ultrasonographer experienced in rodent imaging, using a Toshiba PowerVision 6000 and a 15 MHz transducer. An identical zoom mode depth was always used in order to allow adequate calibration for off-line analysis.

Short-axis two-dimensional (2D) echocardiographic images acquired at the mid-papillary and apical levels of the left ventricle (LV) were stored as digital loops. Frame acquisition rates using the loop mode reached 100 MHz, allowing excellent temporal resolution for 2D analysis. At the same anatomic levels, short-axis M-mode images were obtained with a sweep speed of 100 mm s^{-1}. Echocardiographic studies were performed after the surgical procedure at days 7 and 21 and again at day 42 immediately before killing the animals.

Echocardiographic analysis

Ultrasound analyses were performed by a single researcher experienced in rodent echocardiography, and analyzed by two observers without knowledge of the treatment of each animal, as recently described (Rohde *et al*., 1999). From 2D short-axis imaging, endocardial borders were traced at end systole and end diastole utilizing a prototype off-line analysis system (NICE, Toshiba Medical Systems, The Netherlands). Measurements were performed at the mid-papillary muscle level and at the apical third of the ventricle at the maximal 2D diameter. The end-systolic (smallest) and end-diastolic (largest) cavity areas (ESA and EDA, respectively) were determined. Using the ESA and EDA, fractional area changes were calculated at both levels as ((EDA−ESA)/EDA). From two-dimensionally targeted M-mode tracings, end-diastolic diameter and end-systolic diameter were measured. Fractional shortening (FS) was calculated.

Sample collection, determination of infarct size, and ventricular remodeling

After echocardiographic measurements, hearts were excised and dissected into atria, right and left ventricles including septum. The LV was cut into three transverse sections: apex, middle ring, and base as previously reported (Bauersachs *et al*., 2001). From the middle ring, 5 μm sections were cut and stained with picrosirius red. The boundary lengths of the infarcted and noninfarcted endocardial and epicardial surfaces were traced with a planimeter digital image analyser. Infarct size (fraction of the infarcted LV) was calculated as the percentage of length of circumference.

Collagen content

Picrosirius red polarization microscopy was performed for detection of interstitial collagen according to a modified method of Junqueira (Ducharme *et al*., 2000). Type I and III collagen was identified under illumination with polarized light (Junqueira *et al*., 1979). Sections from a subset of 10 mice that survived the 42-day protocol were chosen with similar MI sizes. Sections were visualized under polarized light, photographed with the same exposure time for each section, and collagen content measured as previously described (Ducharme *et al*., 2000).

Biochemical and molecular measurements

After euthanasia, blood samples were collected for determination of serum glucose (glucometer elite sensor, Bayer, Leverkusen, Germany) and AST. RNA isolation from basal myocardium was performed as previously described (Frantz *et al*., 2001). After the synthesis of cDNA with random hexamers (Superscript, Invitrogen, Karlsruhe, Germany), a real-time PCR was performed (iCycler, BioRad, Munich, Germany) with commercially available TaqMan probes for 18S and murine interleukin 1β (IL-1β), tumor necrosis factor (TNF), and endothelin-1 (Applied Biosystems, Foster City, CA, U.S.A.). PCR parameters were as recommended for the TaqMan universal PCR master mix kit (Applied Biosystems, Foster City, CA, U.S.A.). RNA samples were normalized to 18S rRNA.

Vascular reactivity studies

The descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue (at least $n=4$ in each group) as described elsewhere (Bauersachs *et al*., 1999). The aorta was cut into rings (3–4 mm in length) and mounted in an organ bath (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurement. The rings were equilibrated for 30 min under a resting tension of 1.25 g in oxygenated (95% O_2; 5% CO_2) Krebs–Henseleit solution (NaCl 118 mmol l^{-1}, KCl 4.7 mmol l^{-1}, $MgSO_4$ 1.2 mmol l^{-1}, $CaCl_2$ 1.6 mmol l^{-1}, KH_2PO_4 1.2 mmol l^{-1}, $NaHCO_3$ 25 mmol l^{-1}, glucose 12 mmol l^{-1}; pH 7.4, 37°C). Rings were repeatedly contracted with KCl (100 mmol l^{-1}) until reproducible responses were obtained. Thereafter, the rings were

preconstricted with phenylephrine (1 μmol l^{-1}), and the relaxant response to the cumulative application of acetylcholine (ACh) and sodium nitroprusside (SNP) was assessed.

Statistical analysis

All replicate data are expressed as mean and standard error of mean. Absolute differences among groups were compared using a two-way ANOVA adjusted by the Fisher rule. Statistical significance was achieved when two-tailed $P<0.05$. Statistical analyses were carried out using StatView statistics program (Abacus Concepts, Inc., Berkley, CA, U.S.A.).

Results

Mortality, organ weights, and serum chemistry

In all, 100 animals underwent coronary artery ligation: 55 animals (55%) died before randomization at day 7. Mortality was not different during the subsequent course of the study (four animals died during induction of anesthesia for echocardiography; another four animals (two placebo animals and two pioglitazone animals, $P=$NS) died in their cages). Death was suspected to be attributable to heart failure and arrhythmias. Animals without echocardiographic and histological signs of MI were classified as shams ($n=9$). Animals with a histological infarct size below 30% were excluded from further analyses (six placebo and six pioglitazone animals, $P=$NS).

Body weights at baseline and at 6 weeks were similar among the groups (Table 1). Heart weights after MI were not affected by pioglitazone treatment. Infarct size determined 6 weeks after MI was comparable in both groups (placebo vs pioglitazone, 45.7 ± 9.4 vs $45.7\pm6.7\%$, $P=$NS). After 6 weeks, glucose levels were not different between the groups (see Table 1). TZDs are known to reduce triglyceride levels significantly (Ikeda *et al*., 1990; Tsuji *et al*., 2001). Indeed, in our study, pioglitazone-treated mice with MI had significantly lower triglyceride levels than placebo-treated animals (pioglitazone vs placebo, $n=8$, 79.8 ± 12.2 vs 50.8 ± 5.8 mg dl^{-1}, $P<0.05$).

Echocardiographic measurements

After an initial echocardiography at the time of randomization at week 1, animals underwent follow-up echocardiography at weeks 3 and 6. All measurements were recorded at two levels: at the apical level, which shows changes within the infarcted region, and at the mid-papillary level, which shows changes in the dimensions of the surviving uninfarcted myocardium. Figure 1 shows representative examples of M-mode images at the papillary level of infarcted and uninfarcted mice 42 days after surgery. There were no significant changes in heart rate in either group at all time points. As expected, infarcted ventricles tended to progressively dilate over time with significant larger EDA and ESA as well as distances 3 and 6 weeks after MI (see Figure 2 and Table 2). However, MI animals treated with either placebo or pioglitazone exhibited no differences over time.

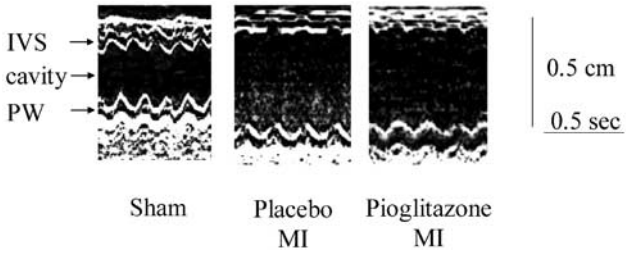

Figure 1 Murine echocardiographic analyses. Representative end-diastolic M-modes of the papillary level of a sham-operated or an infarcted placebo/pioglitazone-treated animal (PW: posterior wall; IVS: interventricular septum). The infarcted animals show enlargement of the LV cavity when compared to the sham-operated animal. No difference could be noted by pioglitazone treatment.

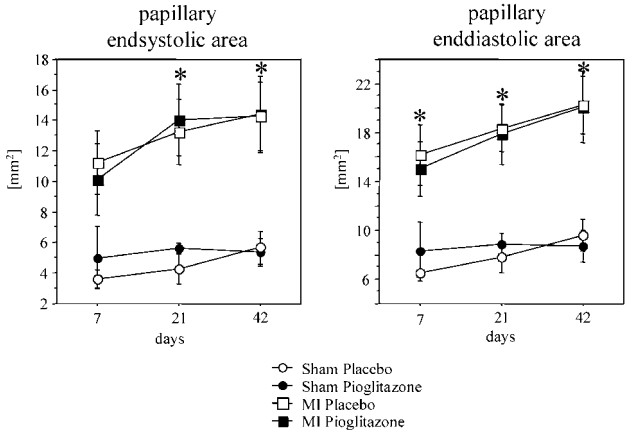

Figure 2 Left ventricular dilatation following MI. The development of left ventricular dilatation on the papillary 2D level over 6 weeks is illustrated. MI animals dilate significantly more over time when compared to sham animals. Treatment with pioglitazone did not affect left ventricular dilatation (*$P\leqslant0.05$, sham vs MI).

Table 1 Organ weights and serum chemistry

	Sham Placebo	*Sham Pioglitazone*	*MI Placebo*	*MI Pioglitazone*
n	5	4	6	10
MI size (%)			45.7 ± 9.4	45.7 ± 6.7
Body wt, baseline (g)	24.2 ± 0.9	26.3 ± 0.8	25.3 ± 0.7	24.4 ± 04
Body wt, 6 weeks (g)	25.0 ± 0.3	26.0 ± 0.4	25.8 ± 0.9	25.2 ± 0.4
LV wt (mg)	97 ± 16	86 ± 3	104 ± 7	98 ± 4
Glucose (mmol l^{-1})	11.0 ± 1.6	12.3 ± 2.0	9.2 ± 1.2	10.2 ± 1.0

Data are mean$\pm$s.e.m., *n* indicates the number of animals studied. LV: left ventricle; wt: weight.

Table 2 Echocardiographic measurements, week 6

	Sham Placebo	*Sham Pioglitazone*	*MI Placebo*	*MI Pioglitazone*
n	5	4	6	10
Heart rate (bpm)	417±29	431±78	443±22	422±34
Papillary				
ESA (mm^2)	5.70±1.07	5.38±0.90	14.40±2.48*	14.79±2.89*
EDA (mm^2)	9.60±1.29	8.70±1.28	20.26±2.38*	20.36±3.36*
2D FS (%)	41.4±4.8	38.3±4.0	30.6±5.2	28.5±7.2
ESD (mm)	2.58±0.24	2.25±0.41	4.72±0.68*	4.10±0.40*
EDD (mm)	3.68±0.33	3.38±0.38	5.68±0.46*	5.15±0.39*
FS (%)	30.2±3.2	34.2±5.5	18.7±5.4	21.3±2.8*
Apical				
ESA (mm^2)	6.80±0.58	5.13±1.66	14.70±2.57*	17.64±3.05*
EDA (mm^2)	10.80±1.16	9.25±1.84	19.15±2.21*	22.43±3.23*
2D FS (%)	34.0±7.3	46.3±8.1	24.7±6.1	23.2±3.1*
ESD (mm)	2.90±0.51	2.68±0.41	4.95±0.66*	4.53±0.60*
EDD (mm)	4.14±0.53	4.00±0.44	5.82±0.50*	5.80±0.33*
FS (%)	32.2±5.2	34.2±4.1	16.2±3.7	24.4±8.7

Data are mean±s.e.m., *n* indicates the number of animals studied. EDA: end-diastolic area; ESA: end-systolic area; FS: fractional shortening; 2D: two-dimensional; ESD: end-systolic diameter; EDD: end-diastolic diameter (*$P<0.05$ sham vs MI).

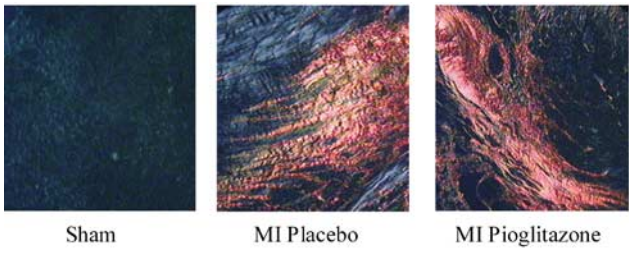

Figure 3 Collagen following MI. Representative examples of picrosirius red stained hearts under polarized light 6 weeks after MI.

Collagen analysis, proinflammatory cytokines, and endothelin-1

There was no significant difference in collagen content between the treatment groups in either the septum (collagen volume fraction, placebo vs pioglitazone, 6.30±1.68 vs 4.43±1.32%, $P=$NS) or in the scar (collagen volume fraction, placebo vs pioglitazone, 74.57±6.61 vs 86.64±5.13%, $P=$NS) (see Figure 3). Moreover, since glitazones are known to inhibit proinflammatory cytokines, myocardial TNF and IL-1β were measured by real-time PCR. Treatment did not influence the cytokine content (MI placebo vs MI pioglitazone, TNF: ΔCT 11.2±1.78 vs 14.48±0.51, $P=$NS, IL-1β: ΔCT 12.47±0.26 vs 12.74±0.27, $P=$NS). Also, endothelin-1 expression was not changed by pioglitazone treatment (MI placebo vs MI pioglitazone, ΔCT 10.9±1.53 vs 11.3±1.28, $P=$NS).

Vascular reactivity in aortic rings

Contraction induced by phenylephrine was not different between the treatment groups. ACh- and SNP-induced relaxation was not affected by treatment with pioglitazone (data not shown).

Discussion

The major finding of this study is the lack of effect of pioglitazone treatment on mortality, left ventricular remodeling, cytokine expression, collagen content, and endothelial function in mice with chronic MI despite a significant triglyceride-lowering effect.

Yet, previous studies revealed effects of TZDs on several pathways of central importance for the development and progression of heart failure: PPARγ activation inhibits TNF production in cardiomyocytes after stimulation with lipopolysaccharides (Takano *et al*., 2000). Indeed, the activation of TNF has been linked to congestive heart failure: The expression of TNF mRNA and protein is elevated in patients and in animal models with advanced heart failure due to different etiologies (Testa *et al*., 1996; Torre-Amione *et al*., 1996). Genetically engineered mice that overexpress TNF exhibit progressive cardiac hypertrophy and ultimately heart failure with many characteristics of human cardiomyopathy (Kubota *et al*., 1997; Bryant *et al*., 1998). Moreover, systemic infusions of recombinant TNF yielding blood concentrations of TNF seen in patients with advanced heart failure depressed left ventricular function and caused left ventricular dilatation (Bozkurt *et al*., 1998). Therefore, PPARγ activation was expected to ameliorate left ventricular dilatation by an inhibition of TNF production. However, neither a reduction of TNF or IL-1β expression nor an attenuation of LV remodeling was observed in our study. Thus, the attenuation of proinflammatory cytokine production does not seem to be effective in the model employed here.

Moreover, TZDs can inhibit endothelin production (Buchanan *et al*., 1995). In patients as well as in animal models of heart failure, endothelin plasma levels are markedly elevated (McMurray *et al*., 1992), and correlate with the severity of heart failure. Indeed, long-term treatment with endothelin receptor antagonists in rats with heart failure following MI significantly prolonged survival of the animals. This effect was associated with improvement of hemodynamics and left

ventricular remodeling (Fraccarollo *et al*., 1997). However, endothelin expression was not changed in our study and therefore does not seem to be functionally important for the data reported here.

Furthermore, activation of PPARγ reduced cardiac hypertrophy in a stretch model *in vitro* (Yamamoto *et al*., 2001) and in a pressure overload model *in vivo* (Asakawa *et al*., 2002) at least partially through the nuclear factor kappa B (NF-κB) pathway. Yet, heart weights in our study were not different between the placebo and pioglitazone groups, suggesting that LV hypertrophy was not affected by pioglitazone.

In addition, TZDs could have potential effects on MI size: In an *in vivo* ischemia/reperfusion model, pretreatment with rosiglitazone (Yue *et al*., 2001) and a number of other PPARγ activators (Wayman *et al*., 2002) reduced infarct size and improved contractile dysfunction. Since westarted treatment 1 week after MI, effects of TZDs on MI size can be excluded. Nevertheless, an attenuation of MI size by an early treatment start could be of central importance for the development of heart failure.

Finally, besides beneficial effects on cardiac myocytes, PPARγ activation exerts marked vasoprotection: Glitazones inhibit the activation and radical production of macrophages, the proliferation of smooth muscle cells, and the formation of the potent vasoconstrictor endothelin in endothelial cells (Buchanan *et al*., 1995; Delerive *et al*., 1999). However, in our model, we did not observe any effect of pioglitazone on aortic endothelial function.

While writing this manuscript, Shiomi *et al*. (2002) published a study demonstrating protective effects of pioglitazone treatment on left ventricular dilatation and heart failure in the same model of chronic murine MI used here. However, there are important differences in the study design: First, different mouse strains were used. Whereas C57Bl6 mice were used in our study, Shiomi *et al*. used CD-1 mice. Thus, the effects of TZDs could be strain dependent. Second, whereas we gave pioglitazone by gavage, Shiomi *et al*. used a dietary supplement allowing different peak and steady-state concentrations. Third, different pioglitazone doses were used: Whereas Shiomi *et al*. used a dose of $3\,mg\,kg^{-1}$ bodyweight, we used $20\,mg\,kg^{-1}$ bodyweight, a dose that is known to have full glucose-lowering activity in mice. Finally, in the study of Shiomi *et al*. infarct sizes were extensive (on average 59%) and treatment was started 6 h after MI. Therefore, protective effects might only be observed in mice with extensive MI when treatment is started early after MI. Moreover, Lygate *et al*. (2003) treated rats with the TZD rosiglitazone for 8 weeks following MI. According to our results, they did not observe modulation of LV remodeling. However, rosiglitazone treatment was associated with increased mortality. In summary, our and other data indicate that the results obtained by Shiomi *et al*. have to be interpreted with caution. Protective effects of pioglitazone on LV remodeling after MI cannot be uniformly observed.

Clinical implications

Glitazones have been implicated in beneficial and detrimental signaling pathways of heart failure. While we could not detect any detrimental effects of TZD treatment when started 1 week after MI in mice, even protective effects in animals with large MI have been observed when treatment was started early (Shiomi *et al*., 2002). Taken together, these data suggest that the use of TZDs in heart failure might be safe. On the other hand, Lygate *et al*. (2003) found an increased mortality in rats treated with TZDs following MI. Moreover, in humans, glitazones are known to cause fluid retention (Tang *et al*., 2003). They are therefore not recommended in patients with heart failure (Kaplan *et al*., 2001). Thus, although we did not detect an increased rate of fluid retentions in the animals with pioglitazone treatment, one should be cautious to use TZDs in patients with heart failure. Indeed, TZDs might have species-specific effects and the mouse model might not be totally appropriate to test this treatment options. Therefore, additional animal studies using different species should be performed.

In conclusion, long-term treatment with the PPARγ activator pioglitazone had no detrimental effects on left ventricular remodeling, fluid retention, and survival in chronic experimental murine MI. Nevertheless, further experimental trials are warranted to clarify the effects of glitazones in heart failure.

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