Effect of Melatonin on Blood Pressure and Nitric Oxide Generation in Rats With Metabolic Syndrome

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Summary

Melatonin, a multitasking indolamine, seems to be involved in a variety of physiological and metabolic processes *via* both receptor-mediated and receptor-independent mechanisms. The aim of our study was to find out whether melatonin can affect blood pressure (BP), nitric oxide synthase (NOS) activity, eNOS and nNOS protein expressions in rats with metabolic syndrome (SHR/cp). Rats were divided into four groups: 6-week-old male WKY andSHR/cp and age-matched WKY and SHR/cp treated with melatonin (10 mg/kg/day) for 3 weeks. BP was measured by tail-cuff plethysmography. NOS activity, eNOS and nNOS protein expressions were determined in the heart, aorta, brain cortex and cerebellum. MT$_1$ receptors were analyzed in the brain cortex and cerebellum. In SHR/cp rats, BP was decreased after melatonin treatment. In the same group, melatonin did not affect NOS activity and eNOS protein expression in the heart and aorta, while it increased both parameters in the brain cortex and cerebellum. Interestingly, melatonin elevated MT1 protein expression in the cerebellum. Neuronal NOS protein expression was not changed within the groups. In conclusion, increased NOS activity/eNOS upregulation in particular brain regions may contribute partially to BP decrease in SHR/cp rats after melatonin treatment. Participation of MT$_1$ receptors in this melatonin action may be supposed.

Key words

Melatonin • MT$_1$ receptors • Metabolic syndrome • Nitric oxide • Heart • Aorta • Brain

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Introduction

Melatonin, a multitasking indolamine (*N*-acetyl-5-methoxytryptamine), shows potent receptor-dependent and -independent actions, which may participate on regulation of both blood pressure and metabolic alterations (Pechanova *et al.* 2014). Receptor-dependent effects are mediated predominantly through MT$_1$ and MT$_2$ G-protein coupled receptors. The pleiotropic receptor-independent effects of melatonin with a possible impact on blood pressure and risk factors of metabolic syndrome involve reactive oxygen species scavenging and activation/overexpression of several antioxidant enzymes or protecting them from oxidative damage. Acting as a direct scavenger, melatonin is able to neutralize different free radicals, mainly superoxide anion and hydroxyl radical (Favero *et al.* 2014, Pechanova *et al.* 2015, Alonso-Alconada *et al.* 2013). Indirect antioxidant actions of melatonin reside in the stimulation of gene expressions and activation of superoxide dismutase, catalase, glutathione peroxidase (Tomas-Zapico and Coto-Montes 2005) and improvement of mitochondrial efficiency (Acuna-Castroviejo *et al.* 2001). Since attenuation of the free radical burden in the central nervous system (CNS) was shown to attenuate hypertension (Kojsova *et al.* 2006), the antioxidant nature

of melatonin may act beneficially particularly in the brain – the tissue highly susceptible to the increased oxidative load (Pechanova *et al.* 2006). Melatonin has been shown to be protective in CNS on several different levels: it reduces free radical burden, improves endothelial dysfunction, reduces inflammation and shifts the balance between the sympathetic and parasympathetic system in favor of the parasympathetic one (Tan *et al.* 2007).

In the CNS, the relation of melatonin regarding its effect on nitric oxide synthase (NOS) activity and/or NOS isoforms expression, is rather complex. This indolamine and its derivates inhibit neuronal NOS (nNOS) and inducible NOS (iNOS), while the effect on endothelial NOS (eNOS) is less clear. Melatonin inhibits iNOS through nuclear factor kappa B (NF-κB)-dependent signaling pathway (Mohan *et al.* 1995, Reiter *et al.* 2000, Mauriz *et al.* 2013), and eNOS *via* modification of Ca^{2+}-calmodulin complex (Pozo *et al.* 1997). Neuronal NOS is activated by calcium similarly to eNOS and analogous mechanism in melatonin-nNOS interaction in the brain may be supposed.

Recently it was found that simultaneous application of melatonin with lisinopril or amlodipine had the normalizing effect on metabolic parameters in patients with arterial hypertension (Shatilo *et al.* 2012). Obese spontaneously hypertensive SHR/NDmcr-cp/cp (SHR/cp) rats, in which the leptin receptor gene has been knocked out, represent a useful model for monitoring parameters associated with metabolic syndrome, since this respective model develops hypertension, obesity, hyperlipidemia, hyperinsulinemia and hyperglycemia (Michaelis *et al.* 1984, Striffler *et al.* 1998, Nagase and Fujita 2010). Moreover, several studies also reported elevated circulating levels of oxidative stress markers and disturbance in the nitric oxide/cyclic guanosine monophosphate (cGMP) pathway in SHR/cp rats (Yamaguchi *et al.* 2006, Kagota *et al.* 2006).

Thus, the aim of our study was to find out whether melatonin can affect blood pressure in SHR/cp rats and induce related changes in NOS activity and eNOS and nNOS protein expressions. Participation of brain MT_1 receptors in melatonin action was studied as well.

Methods

Chemicals and drugs

All of the chemicals used were purchased from Sigma Chemicals Co. (Germany), when not specified.

Animals and treatment

The experiments were carried out in male normotensive Wistar-Kyoto rats (WKY) and hypertensive rats with metabolic syndrome – SHR/ND mcr-cp (cp/cp) (SHR/cp) housed in a room with a maintained temperature (22±2 ^{o}C), relative humidity (55±10 %), and 12-h light/dark cycle. The animals had free access to standard lab chow (pelleted ST-1 diet) and water *ad libitum*. The SHR/cp rats were obtained from Charles River US laboratories. All procedures and experimental protocols were approved by the Ethical Committee of the *Institute of Normal and Pathological Physiology SAS*, and conformed to the *European Convention on Animal Protection* and *Guidelines on Research Animal Use*.

Rats were divided into four groups: control 6-week-old male WKY and SHR/cp and age-matched WKY and SHR/cp rats orally treated with melatonin (10 mg/kg/day) for 3 weeks (n=6 in each group). Systolic blood pressure was measured by tail-cuff plethysmography every week. At the end of experiment the animals were sacrificed and body weight (BW), heart weight (HW), and tibia length (TL) were determined. Samples of the heart left ventricle (LV), aorta, brain cortex and cerebellum were used for the determination of NOS activity and Western blot analysis.

Total NOS activity

Total NOS activity was determined in crude homogenates of LV, aorta, brain cortex and cerebellum by measuring L-[^{3}H]citrulline formation from L-[^{3}H]arginine (MP Biochemicals, California, USA) as described elsewhere (Pechanova *et al.* 1997, Bredt and Snyder 1990). NOS activity was expressed as pkat/min per gram of protein.

Western blot analysis

Samples of the tissues (LV, aorta, brain cortex and cerebellum) were probed with a polyclonal rabbit anti-endothelial NOS, anti-neuronal NOS antibodies and antibodies for MT1A-R (Santa Cruz, USA) as described elsewhere (Bourcier *et al.* 1997). Antibodies were detected using a secondary peroxidase-conjugated anti-rabbit antibody (Santa Cruz, USA). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analyzed densitometrically using Photo-Capt V.99 software.

Statistical analysis

Results are expressed as means ± SEM. One-way ANOVA and Duncan´s test were used for statistical

analysis. P<0.05 value was considered statistically significant.

Results

### Weight ratios and systolic blood pressure (SBP)

In SHR/cp rats body weight, heart weight and relative heart weight (calculated as the ratio of HW to TL) were significantly higher in comparison to control WKY rats. Melatonin treatment did not affect these parameters in any rat strain (Table 1).

Table 1. Effects of melatonin treatment on body weight (BW), heart weight (HW) and heart weight/tibia length HW/TL ratio.

	BW (g)	HW (mg)	HW/TL (mg/mm)
WKY	249±5	814±17	22±0.4
WKY + MEL	250±4	819±21	23±0.5
SHR-cp	315±16*	878±29*	26±0.6*
SHR-cp + MEL	323±16*	867±20*	26±0.7

WKY – Wistar Kyoto rats, SHR-cp – rats with metabolic syndrome, MEL – melatonin treatment (10 mg/kg/day). Data are means ± SEM; significant differences *P<0.05 compared with WKY rats.
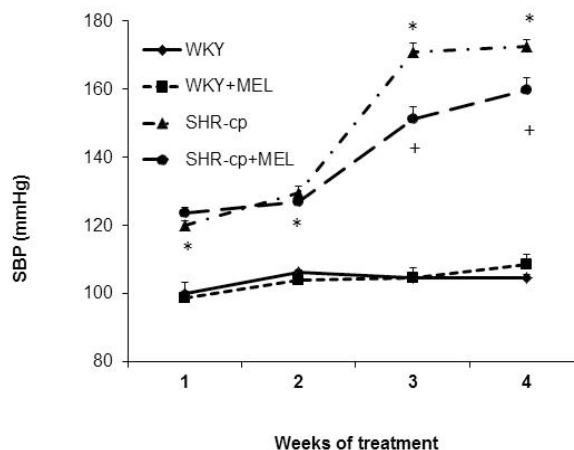
Fig. 1. Effect of melatonin (MEL) treatment on systolic blood pressure (SBP) in normotensive Wistar-Kyoto rats (WKY) and hypertensive rats with metabolic syndrome (SHR-cp). * p<0.05 compared with WKY, + p<0.05 compared with SHR-cp.

Melatonin treatment did not affect SBP of normotensive WKY controls. SBP of SHR/cp rats was significantly higher during the whole experiment and melatonin treatment decreased it by 10 % already after the second week of the treatment. This decrease persisted till the end of the treatment in comparison to age-matched untreated rats (159.6 vs.172.5 mm Hg, p<0.05) (Fig. 1).
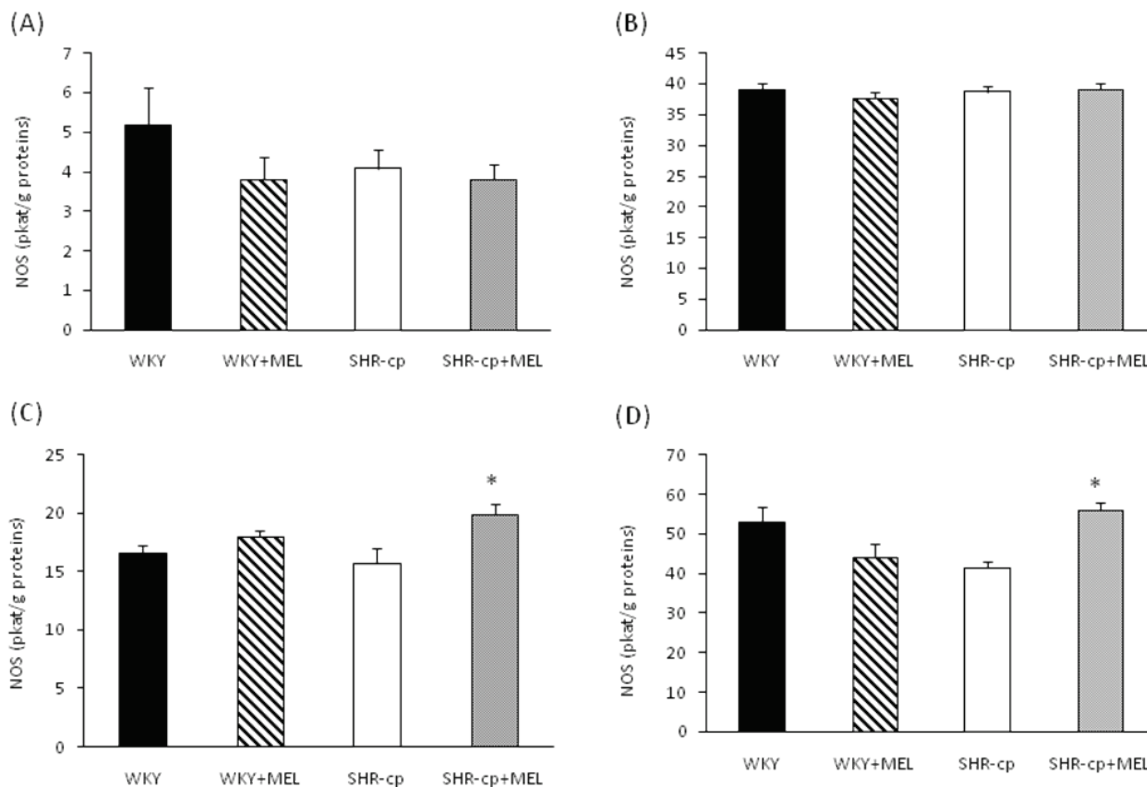
Fig. 2. Effect of melatonin (MEL) treatment on nitric oxide synthase (NOS) activity in the left ventricle (LV) **(A)**, aorta **(B)**, brain cortex **(C)** and cerebellum **(D)** of Wistar-Kyoto rats (WKY) and rats with metabolic syndrome (SHR-cp). * p<0.05 compared with respective control groups without MEL treatment.

Total NOS activity

No significant changes in total NOS activity were found in the tissues of WKY and SHR/cp rats. After melatonin treatment, total NOS activity was not changed significantly in the LV and aorta within the groups (Figs 2A and 2B). However, in SHR/cp rats melatonin treatment increased total NOS activity in brain cortex by 25 % (p<0.05) (Fig. 2C) and in cerebellum by 35 % (p<0.05) (Fig. 2D). There were no changes in total NOS activity after melatonin treatment in the WKY group.

Western blot analysis

Melatonin treatment increased eNOS protein expression in the brain cortex and cerebellum of both

WKY (by 25 % and 17 %, respectively) and SHR/cp rats (by 36 % and 15 %, respectively) (p<0.05). On the other hand, eNOS protein expression in the LV and aorta remained unchanged (Figs 3A, 3B, 3C and 3D).

No changes in nNOS protein expression were found either in the LV and aorta or brain cortex and cerebellum within the groups (data not shown).

In WKY rats melatonin treatment increased protein expression of MT_1 receptors in the brain cortex by 15 % and cerebellum by 22 % (p<0.05). In SHR/cp rats melatonin treatment increased significantly protein expression of MT_1 receptors in the cerebellum by 18 % (p<0.05). Nevertheless, there was a tendency to elevation in the brain cortex as well (Figs 4A and 4B).
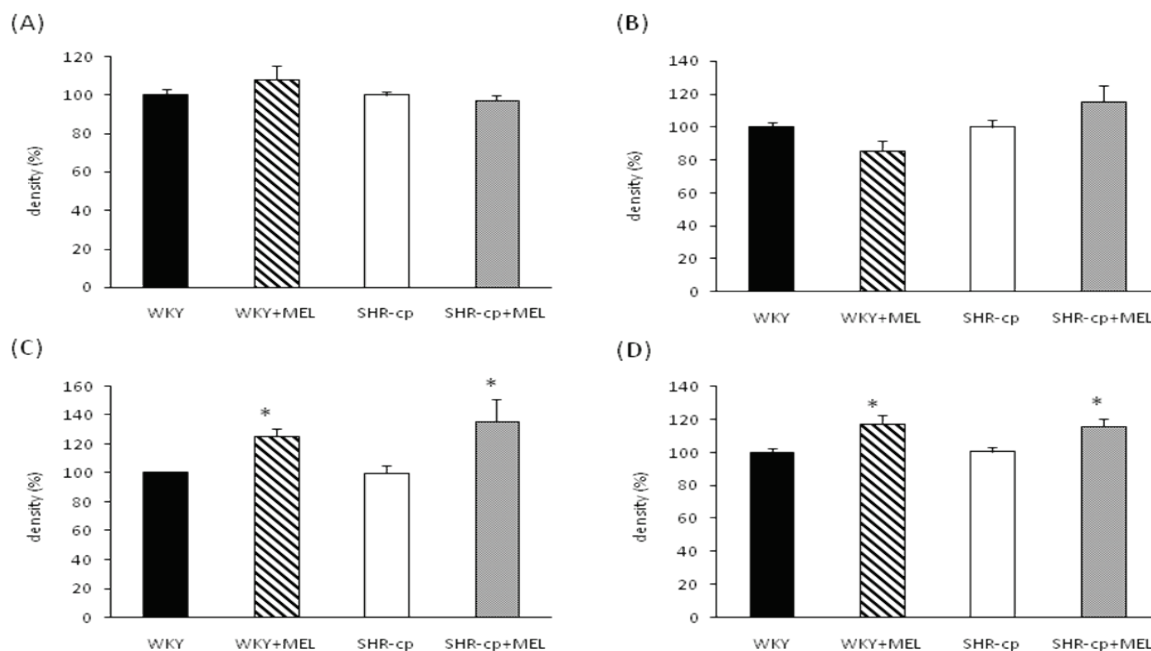
Fig. 3. Effect of melatonin (MEL) treatment on expression of endothelial nitric oxide synthase (eNOS) in the left ventricle (LV) **(A)**, aorta **(B)**, brain cortex **(C)** and cerebellum **(D)** of Wistar-Kyoto rats (WKY) and rats with metabolic syndrome (SHR-cp). * p<0.05 compared with respective control groups without MEL treatment.
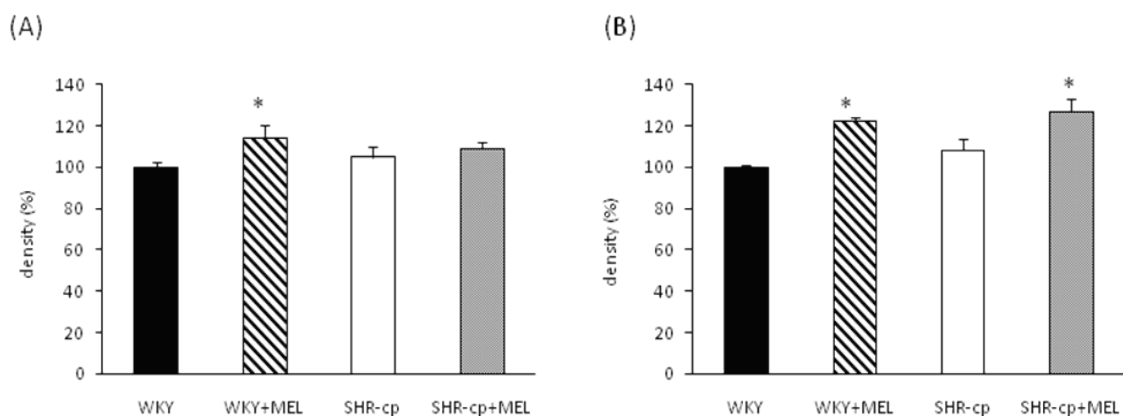
Fig. 4. Effect of melatonin (MEL) treatment on expression of melatonin receptors type 1 (MT_1) in the brain cortex **(A)** and cerebellum **(B)** of Wistar-Kyoto rats (WKY) and rats with metabolic syndrome (SHR-cp). * p<0.05 compared with respective control groups without MEL treatment.

Discussion

The SHR/NDmcr-cp rat has a genetic background from the spontaneously hypertensive rat (SHR) and carries nonsense mutation of leptin receptor derived from the obese Koletsky rat. SHR/NDmcr-cp rats develop severe hypertension compared with age-matched normotensive control WKY. Their systolic blood pressure reaches 200 mm Hg at 12 weeks of age (Michaelis *et al*. 1984). Moreover, they are characterized by increased body and heart weights (Kagota *et al*. 2009, Janega *et al*. 2014), which was clearly confirmed by our study (Table 1). Furthermore, we have documented increased relative heart weight (normalized by tibia length) in this respective model. Fluctuations in body weight as occur with obesity make body weight an unreliable reference for normalizing heart weight. We therefore used tibia length, which remains constant with overweight, for calculating relative heart weight. Melatonin treatment did not affect either body weight or relative heart weight within the groups. However, in SHR/cp rats melatonin decreased systolic blood pressure significantly already after the second week of the treatment and SBP reduction persisted till the end of the experiment. Similar blood pressure reduction we have seen in melatonin-treated SHR rats (Pechanova *et al*. 2007, Simko *et al*. 2009) and it was also reported in other experimental studies (Huang *et al*. 2013, Siew-Keah *et al*. 2014).

We have also analyzed the contribution of NOS activity in the blood pressure reduction after melatonin treatment. Interestingly, in SHR/cp rats melatonin did not affect NOS activity and eNOS protein expression in the left ventricle and aorta, while it increased these parameters in the brain cortex and cerebellum. We admit that peripheral mechanisms of blood pressure regulation are much more thoroughly considered than those at the level of central nervous system, involving predominantly the relative dominance of sympathetic and renin-angiotensin systems (Brunová *et al*. 2015). In SHR, the acute administration of melatonin reduced blood pressure along with norepinephrine levels (K-Laflamme *et al*. 1998). The acute administration of melatonin to normotensive rats also reduced blood pressure, heart rate along with the reduction of serotonin levels in corpus striatum and hypothalamus (Chuang *et al*. 1993). Moreover, in these experiments, the effect of melatonin on blood pressure and heart rate was abolished by spinal transection or bilateral vagotomy, suggesting the involvement of sympathetic inhibition or parasympathetic stimulation by melatonin (Chuang *et al*. 1993). Chronic administration of melatonin, similarly like N-acetylcysteine, decreased blood pressure and heart rate and improved the chronotropic response to isoproterenol, in association with the inhibition of sympathetic activity (Girouard *et al*. 2003). In young healthy men, melatonin reduced pulsatile index and systolic blood pressure along with norepinephrine levels (Arangino *et al*. 1999) and reduced blood pressure and pulse wave velocity in association with the attenuation of sympathetic tone (Yildiz *et al*. 2006). These sympatholytic effects of melatonin may be involved in the blood pressure reducing effect of melatonin seen in SHR (Pechanova *et al*. 2007), L-NAME hypertensive rats (Paulis *et al.* 2010a, Paulis *et al*. 2010b), healthy volunteers (Arangino *et al*. 1999) or patients with essential hypertension (Simko and Pechanova 2009). The association of melatonin administration with sympatholytic effects may suggest a serious role of the central effects of melatonin on blood pressure regulation.

It is true that central NO can reduce blood pressure through the attenuation of sympathetic activity in hypertensive rats (Zhou *et al*. 2014, Ramachandra *et al*. 2014, Pechanova and Simko 2009). Thus, we hypothesized that melatonin may contribute to blood pressure reduction by increasing brain NOS activity with the attenuation of sympathetic tone also in our experimental study. The question arises which NOS isoform may contribute mostly to this blood pressure reduction. Although Guo *et al.* (2009) suggested a predominant role of nNOS in this process, other studies preferred increased eNOS expression in both sympathetic activity and blood pressure reduction (Kimura *et al*. 2007, Zhu *et al*. 2016, Kishi *et al* 2001). In accordance with the latter studies, in our experiment with SHR/cp rats, melatonin treatment increased brain eNOS, while it had no effect on nNOS protein expression. Thus, we assume that in the SHR/cp rats melatonin may upregulate rather brain eNOS with a decreasing effect on blood pressure.

Moreover, it has been shown that in stress-induced hypertensive rats, melatonin levels in the anterior hypothalamic area were reduced. A microinjection of melatonin into this site reduced blood pressure along with increased GABAergic activity and reduced glutama-tergic activity in the rostral ventrolateral medulla. This effect was prevented by MT_1/MT_2 blockade (Xia *et al*. 2008). Since in SHR/cp rats NOS activity was increased in the brain parts like brain cortex and cerebellum, we

analyzed protein expression of MT_1 receptors in these brain regions. Interestingly, in our study melatonin not only increased brain NOS activity and decreased blood pressure but also elevated MT_1 protein expression. It was hypothesized that the activation of melatonin receptors on endothelial cells would trigger NO production and contribute to blood pressure reduction (Paulis and Simko 2007). It seems, however, that melatonin receptors may regulate blood pressure also through the central mechanisms, since the highest density of melatonin receptors has been shown in the central nervous system (Malpaux *et al.* 2001, Vaněček and Janský 1989, Duncan *et al.* 1989).

Taken together, increased NOS activity and eNOS upregulation in the brain may contribute to blood pressure decrease in SHR/cp rats after melatonin treatment. Participation of MT_1 receptors in melatonin action may be supposed.

Conclusions

For the first time it has been shown, that melatonin may reduce blood pressure in rats with metabolic syndrome by increased production of central nitric oxide. Involvement of eNOS and MT_1 protein expressions in the brain is supposed. However, mechanisms of MT_1-melatonin-NO interplay at the CNS level leading to blood pressure reduction need to be more clarified. The inhibition of sympathetic nerve activity by nitric oxide is suggested.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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