The effect of indapamide on development of myocardial hypertrophy and fibrosis in L-NAME-induced hypertension in rat

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Short title: Indapamide and Myocardial Remodeling

Summary

Aim: The aim of this study was to analyze the effect of indapamide and its combination with ACE inhibitor (captopril) and antioxidant (Provinols™) on both myocardial hypertrophy and fibrosis. **Methods:** Wistar rats were treated with L-NAME (40 mg/kg/day, L); L-NAME plus indapamide (1 mg/kg/day), or captopril (10 mg/kg/day), or Provinols™ (40 mg/kg/day), or combination of indapamide with captopril, and indapamide with Provinols™ for 7 weeks. Blood pressure (BP), LV hypertrophy and fibrosis were determined. The content of collagens type I and III was evaluated morphometrically after picrosirius red staining. **Results:** L-NAME treatment led to increased BP, LV hypertrophy, total fibrosis and relative content of collagens without the change in collagen type I/III ratio. Indapamide and captopril decreased BP, LV hypertrophy and the collagen ratio without affecting total fibrosis, while Provinols™ reduced BP, the collagen ratio and fibrosis without affecting LV hypertrophy. The combinations decreased all the parameters. **Conclusions:** Decrease of LV hypertrophy was achieved by drugs with the best reducing effect on BP, fibrosis reduction was reached by the antioxidant treatment with only partial effect on BP. Thus, the combination of antihypertensive and antioxidant treatment may represent a powerful tool in preventing myocardial remodeling induced by hypertension.

Key words: captopril, indapamide, LV hypertrophy, myocardial fibrosis, red wine polyphenols.

Introduction

Persistently increased blood pressure is one of the risk factors for various cardiovascular diseases and is associated with left ventricular (LV) hypertrophy (Gottdiener *et al.* 1997). Increased myocardial mass is determined by enlargement of cardiac myocytes, which may be accompanied by proliferation of fibroblasts and the expansion of extracellular matrix. The collagen accumulation and the left ventricular hypertrophy develop as the consequences of increased blood pressure and both are considered to participate at myocardial stiffening (Narayan *et al.* 1989, Schraeger *et al.* 1994).

The effect of NO-synthase inhibitor *N*(G)-nitro-L-arginine methyl ester (L-NAME) on left ventricular hypertrophy is disputable. According to some findings, even chronic (6-8 weeks) administration of this NO synthase inhibitor did not cause significant change in the mass of the left ventricle (Arnal *et al.* 1992, Arnal *et al.* 1993, Bartunek *et al.* 2000). Some authors observed hypertrophy development not before 8 weeks (Moreno *et al.* 1996, Takemoto *et al.* 1997) while others described development of left ventricular hypertrophy after 4 weeks of treatment with L-NAME (Bernatova *et al.* 1996, Pechanova *et al.* 1999, Bernatova *et al.* 2000). The effect of L-NAME on myocardial fibrosis has been well described by various authors. It seems that accumulation of collagen in the myocardium is not directly connected with increased blood pressure but rather with the decreased NO synthesis that leads to activation of neurohormonal systems and growth-promoting factors (Moreno *et al.* 1996, Takemoto *et al.* 1997, Pechanova *et al.* 1999, Bernatova *et al.* 2000). It is supposed that NO deficiency rather than hemodynamic changes appears to be crucially involved in fibrous tissue changes of the left ventricle in hypertension induced by L-NAME (Pechanova *et al.* 1999).

Myocardial extracellular matrix is composed of various structural proteins, predominantly of collagens type I and III (Weber 1989). Collagen type I (Col I) provides strength and rigidity,

whereas tissues with large amount of collagen type III (Col III) are characterized by increased elasticity (Marijianowski *et al.* 1995). Recent studies showed that the different collagens accumulation is connected with the development of heart failure, increased passive stiffness and impaired contractile function (Conrad *et al.* 1995, Yamamoto *et al.* 2002). Some authors (Mukherjee & Sen 1990) suppose the ratio of collagen type I/III (Col I/III ratio) to be a more important prognostic factor than the total amount of collagens and it was proved that its increase is associated with dilatation cardiomyopathy (Marijianowski *et al.* 1995) and with post-infarction remodeling of unaffected myocardium of both ventricles (Wei *et al.* 1999). Myocardial hypertrophy as well as fibrosis enlargement may be influenced by various therapeutic agents including antihypertensives. Indapamide, a non thiazide diuretic, has been used for its antihypertensive and blood pressure variability stabilizing effect (Zhang *et al.* 2011). It also was documented to reduce left ventricular hypertrophy and total myocardial fibrosis in stroke prone spontaneously hypertensive rats (SHR) (Contard *et al.* 1993). According to Janega *et al.* (2007) the effect of indapamide in SHR was not dependent on nitric oxide production. Indapamide is often combined with other antihypertensive drugs like angiotensine-converting enzyme (ACE) inhibitors. Captopril, an ACE inhibitor with thiol groups, caused regression of left ventricular hypertrophy, significantly changed Col I/III ratio in SHR and was able to decrease the total amount of collagen in the heart after chronic treatment with L-NAME (Mukherjee & Sen 1993, Pechanova *et al.*1997). Since antioxidant properties of 5-OH indapamide, the major metabolite of indapamide, have been described (Vergely *et al.* 1998), it was hypothesized that the possible antihypertophic and/or antifibrotic properties of indapamide might be supported by antioxidants. Red wine polyphenols with powerful antioxidant effect, have been shown to reduce fibrosis development yet without affecting left ventricular hypertrophy caused by L-NAME treatment (Bernatova *et al.* 2002, Pechanova *et al.* 2004, Hlavackova *et al.* 2009).

The aim of this study was to analyze the effect of indapamide and its combination with captopril or red wine polyphenols (Provinols^{TM}) on left ventricular hypertrophy and collagen type I and type III content in myocardium in the model of experimental NO deficient hypertension.

Methods

All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use. The animals were housed in an air-conditioned room at a stable temperature (22-24 °C) and humidity (45-60%) on a 12:12 hour light/dark cycle and maintained on a standard pellet diet and tap water *ad libitum*. Daily water consumption was estimated one week before the experiment and controlled during the treatment.

Adult 12-week-old male Wistar rats were divided into 8 groups with 6 animals in each group: control group (Con), group treated with 40 mg/kg/day of L-NAME (L); groups receiving L-NAME (40 mg/kg/day) plus indapamide 1 mg/kg/day (LI), or captopril 10 mg/kg/day (LC), or Provinols^{TM} 40 mg/kg/day (LP), or combination of indapamide with captopril (LCI), or combination of indapamide with Provinols^{TM} (LPI). All compounds were dissolved in the drinking water and administered orally for 7 weeks. At the end of the treatment the body weight (BW) and left ventricular weight (LVW) was measured and LVW to BW ratio was calculated (LVW/BW).

The red wine extract dry powder Provinols™ was kindly provided by Mr. D. Ageron (Société Francaise de Distillerie, Vallont Pont d´Arc, France). Provinols™ polyphenols content is known and has been reported (Diebolt *et al.* 2001) as follows (in mg/g of dry powder):

proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14.

Blood pressure measurement

The blood pressure (BP) was measured non-invasively by the tail-cuff plethysmography using the Statham Pressure Transducer P23XL (Hugo Sachs, Germany) every week. The final value was calculated from five successive measurements.

Histology

The hearts were fixed 24 hours in 10 % formalin, routinely processed in paraffin and 5 μm thick sLCIes were stained with hematoxylin and eosin. The slides were evaluated in a Leica light microscope (Leica Systeme, Wetzlar, Germany).

Collagen type I. and III. evaluation

Sirius red F3BA dissolved in a saturated picric acid stains collagens type I and III, which can be distinguished with the use of polarized light, and their content evaluated by computer assisted morphometry is in concordance with the results obtained by immunohistochemistry and evaluation of mRNA levels (Pauschinger *et al.* 1999, Allon *et al.* 2006).

Deparaffinized and rehydrated 5 μm thick sLCIes were stained with a modified technique with picrosirius red as follows: the slides were submerged in 0.2 % phosphomolybden acid for clearing the cytoplasm, then the slides were stained with 0.1 % sirius red F3BA in a saturated water solution of picric acid for 90 min. The slides were washed 2 min in 0.01 N HCl, dehydrated and mounted.

Five randomly selected places on each slide were selected, viewed under polarized light, documented with a digital photographic camera S50 (Canon, Japan) and evaluated with

ImageJ software (National Institute of Health, Bethesda, USA). Threshold values were determined for the particular colors of spectrum, the numbers of pixels of each color were counted and the percentage of the picture's area was calculated.

Statistics

The results were expressed as mean±standard deviation, statistically analyzed by two-way (blood pressure) or one-way ANOVA with Keuls-Neumann test. Values with $p<0.05$ were considered significant.

Results

Left ventricular hypertrophy and blood pressure

Blood pressure of the rats was not significantly different between the groups at the beginning of the experiment. Indapamide and captopril and indapamide together with captopril significantly decreased the blood pressure rise from the second week of treatment when compared with the group receiving L-NAME only. From the fourth week significant decrease of the blood pressure rise was achieved by administration of all tested compounds and their combination (Fig. 1 and 2).

The body weight (BW) was not changed in comparison to the control in any of the groups except for the animals receiving captopril and indapamide with a significant decrease of BW. Left ventricular weight and ventricular weight/body weight ratio were increased significantly in rats receiving L-NAME when compared to control; only administration of Provinols^{TM} caused no significant decrease of these parameters (Tab. 1).

Myocardial fibrosis

Changes of myocardial fibrosis can be shown on histological pictures where red to yellow fibers represent collagen type I, green fibers show collagen type III.

When digitally processed and statistically evaluated it could be seen that administration of L-NAME resulted in a 73% increase of collagen type I content in the myocardium when compared to control ($p < 0.001$). Col I was not reduced by indapamide or captopril in comparison to L-NAME only, but their combination, as well as Provinols^{TM} with and without indapamide resulted in a significant decrease of relative content of collagen type I in myocardium when compared to the L-NAME group ($p < 0.001$ for all mentioned, data not shown).

Collagen type III was increased by 54% after administration of L-NAME ($p <0.05$) and interestingly, the addition of indapamide caused a further increase of Col III when compared to the control ($p < 0.001$ vs. control), but this change was not significant versus the L-NAME group. No other of the tested substances or combinations caused significant changes in collagen type III in comparison to the control or the L-NAME groups (data shown).

The total myocardial fibrosis increased by 63% after chronic administration of L-NAME (compared to Con). Neither indapamide nor captopril were able to influence the effect of L-NAME significantly, however, their combination caused a decrease of total fibrosis by 48%. Significant decrease of total fibrosis was observed after simultaneous administration of Provinols^{TM}, 42% without and 53% with indapamide in comparison to the L-NAME group (Fig. 3).

Collagen type I/III ratio was not changed after chronic administration of L-NAME. All tested compounds added to L-NAME caused a significant decrease of the Col I/III ratio in comparison to both, the control and the L-NAME group ($p < 0.05$ for all groups except Provinols^{TM} + indapamide where $p < 0.001$; data not shown).

Discussion

Experimental hypertension produced by chronic administration of L-NAME resulted in increased blood pressure, left ventricular hypertrophy, increased content of Col I, Col III and total fibrosis in the myocardium, but not in the change of Col I/III ratio. Similar effects on blood pressure, left ventricular hypertrophy and total myocardial fibrosis were reported after chronic administration of L-NAME at even lower doses (Pechanova *et al.* 1999). However, the effect of L-NAME on the two types of collagen and their ratio in the heart has not yet been described.

Regarding the process of fibrosis and myocardial hypertrophy the role of both NO and the actual level of blood pressure is still disputable. Comparable changes in the heart can be seen in spontaneously hypertensive rats that have increased production of NO (Janega *et al.* 2007) and in L-NAME treated rats with NO deficiency. Even administration of L-arginine was not able to prevent the myocardial remodeling in L-NAME treated rats (Simko *et al.* 2005).

In our study all the investigated substances decreased the blood pressure but their effects on the evaluated parameters in the heart tissue were variable. These results are in concordance with those obtained in L-NAME induced hypertension and in SHR obtained by others (Kobayashi *et al.* 2000, Innes *et al.* 1998, Varo *et al.* 2000), as well as in patients with hypertension (Gottdiener *et al.* 1997). This suggests the existence of other important factors influencing the heart muscle that are relatively or absolutely independent on NO or on the actual level of blood pressure.

Administration of indapamide alone causes reduction of the blood pressure, regression of the left ventricular hypertrophy and a decrease in the Col I/III ratio, but does not contribute to the decrease of the total fibrosis and relative content of the collagens in L-NAME induced hypertension. These results differ from those obtained in SHR where indapamide reduces the

total myocardial fibrosis, but has no effect on the collagen type I/III ratio and its effect was independent on NO synthase activity (Janega *et al.* 2007, Nguyen *et al.* 1998).

Myocardial parameters in spontaneously hypertensive rats (Nguyen *et al.* 1998) after captopril administration were similar to those observed after indapamide treatment. Different results, however, can be seen in SHR and in humans after ACE inhibitor (lisinopril) treatment. While lisinopril causes regression of myocardial fibrosis in SHR, in humans it had no effect on the heart hypertrophy (Serafini *et al.* 1998).

Surprisingly, combination of captopril and indapamide is able significantly to reduce the total fibrosis and the Col I content. These results indicate that prevention of myocardial fibrosis and hypertrophy seems to be dependent on more than one pathway and that oxidative stress may be one of the contributing factors. Both indapamide and captopril are known to have antioxidant properties (Kojsova *et al.* 2006), which are probably insufficient to decrease the myocardial fibrosis when administered singly, but are complementary after the combination. The combination of indapamide and captopril was shown to exert additive effects in SHR, increasing NOS activity, eNOS protein expression in the aorta and decreasing conjugated dienes concentration in the kidneys, in contrast with monotherapeutic application (Vranková *et al.* 2009). As for the body weight loss after the combination of indapamide and captopril, this effect of indapamide or captopril has been reported (Machová 1988, Weisinger *et al.* 2009). In our experimental settings it was demonstrated that combination of these drugs potentiated their lowering effect on the body weight which was not significant when given individually.

The multifactorial genesis of the myocardial fibrosis and hypertrophy is also supported by the results obtained by the administration of red wine extract Provinols^{TM}. Singly administration of red wine polyphenols is able to decrease the blood pressure in L-NAME induced hypertension, reduces the relative content of the collagen type I together with the total fibrosis

and the collagen type I/III ratio. Red wine polyphenols are well known to possess antioxidant properties and enhance plasma antioxidant capacity (Leikert *et al.* 2002). They also modulate the activity and expression of NO synthases (Kim *et al.* 2006, Diebolt *et al.* 2001), influence prostaglandins secretion and cyclooxygenases expression (Oak *et al.* 2004) and inhibit the synthesis of MMP-2 (Vergely *et al.* 1998), which is elevated in patients with heart failure (Altieri *et al.* 2003). Presumably, captopril or indapamide cannot affect all these pathways alone as the red wine polyphenols but they gain these abilities after their combination.

On the other hand, red wine polyphenols alone are not able to influence the LV hypertrophy that obviously develops independently on myocardial fibrosis. But the combination with indapamide provides this additional effect and results in general improvement of the described myocardial parameters.

In conclusion, our results indicate that administration of indapamide is able to prevent the increase of blood pressure and the myocardial hypertrophy but has insignificant effect on myocardial fibrosis. This limitation can be avoided by adding captopril or Provinols^{TM} to the treatment. Such combinations have the ability to prevent the myocardial fibrosis possibly through various pathways including the enhancement of antioxidant properties and the influence on nitric oxide synthesis. These results may contribute to the ongoing intense search for the optimal antihypertensive therapy schemes with preventive effect on myocardial remodeling.

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Table 1.

Body weight, left ventricular weight and relative left ventricular weight after 7 weeks of administration of N(G)-nitro-L-arginine methyl ester (L-NAME)

	Body weight: BW (g)	Left ventricular weight: LVW (mg)	Relative left ventricular weight: LVW/BW (mg/g)
Con	341 ± 7	465 ± 29	1.36 ± 0.07
L	349 ± 10	536 ± 20 $^{+}$	1.54 ± 0.06 $^{+}$
LI	334 ± 15	441 ± 17 *	1.35 ± 0.08 *
LC	339 ± 6	408 ± 4 *	1.21 ± 0.03 *
LCI	305 ± 16 *	405 ± 5 *	1.29 ± 0.5 *
LP	352 ± 13	500 ± 24	1.43 ± 0.08
LPI	346 ± 15	420 ± 5 *	1.23 ± 0.07 *

L-NAME (L) and indapamide (LI), or captopril (LC), combination of indapamide and captopril (LCI), or red wine polyphenols (LP), combination of indapamide and red wine polyphenols (LPI). Numbers represent the average value ± SD. $^{+}$ $p < 0.05$ vs. control (Con), * $p<0.05$ vs. L.

Figure legends:

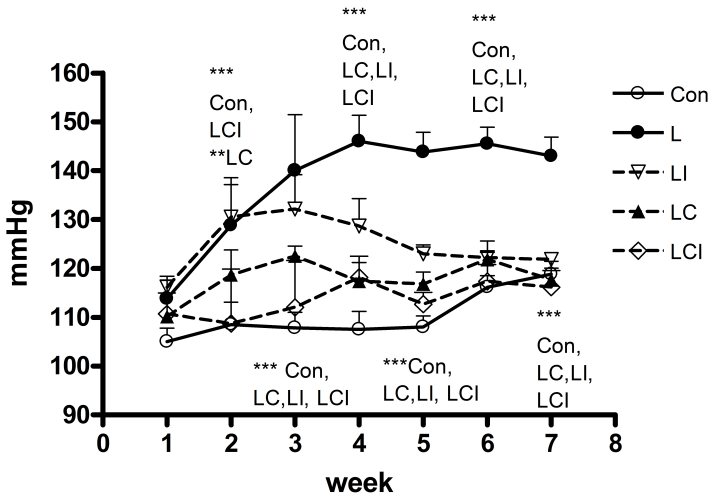

Figure 1: Blood pressure during simultaneous administration of L-NAME (L) and indapamide (LI), or captopril (LC), or combination of indapamide and captopril (LCI) for 7 weeks. Control is labeled as Con. Numbers represent the average value ± SD. * p < 0.05, ** p<0.01, *** p<0.001 vs. L-NAME group (L).

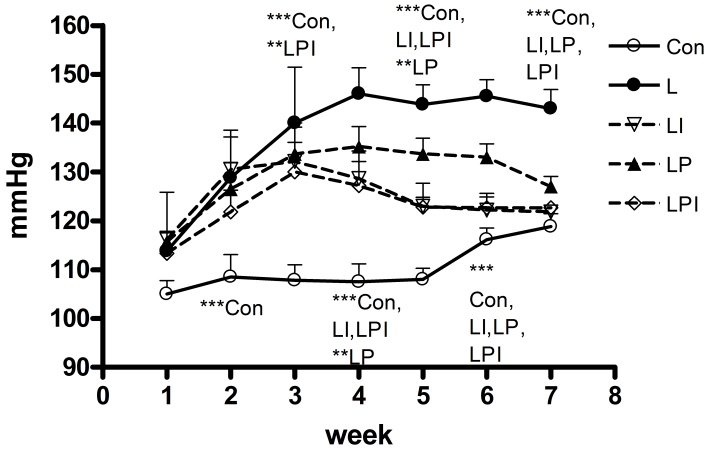

Figure 2: Blood pressure during simultaneous administration of L-NAME (L) and indapamide (LI), or red wine polyphenols (LP), or combination of indapamide and red wine polyphenols (LPI) for 7 weeks. Control is labeled as Con. Numbers represent the average value ± SD. * p < 0.05, ** p<0.01, *** p<0.001 vs. L-NAME group (L).

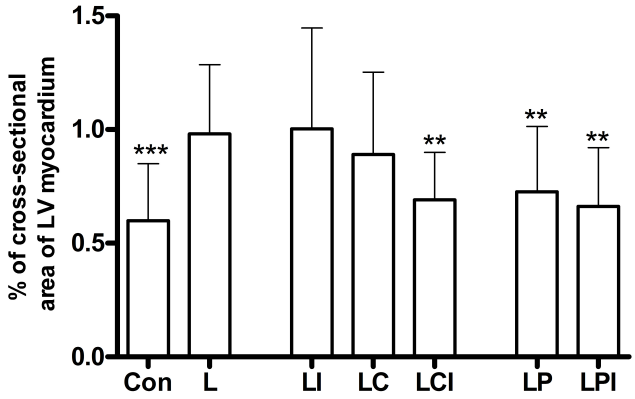

Figure 3: Myocardial fibrosis after 7 weeks of simultaneous administration of L-NAME (L) and indapamide (LI), or captopril (LC), or combination of indapamide and captopril (LCI), or red wine polyphenols (LP), or combination of indapamide and red wine polyphenols (LPI). Control is labeled as Con. Numbers represent the average value ± SD. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ vs. L-NAME group (L).