Leptin Potentiates Endothelium-Dependent Relaxation by Inducing Endothelial Expression of Neuronal NO Synthase

Sebastian Benkhoff, Annemarieke E. Loot, Ina Pierson, Adrian Sturza, Karin Kohlstedt, Ingrid Fleming, Hiroaki Shimokawa, Olaf Grisk, Ralf P. Brandes, Katrin Schröder

Objective—Obesity is associated with hyperleptinemia but it is not clear whether leptin protects vascular function or promotes dysfunction. We therefore studied the consequences of hyperleptinemia in lean mice.

Methods and Results—Wild-type and endothelial NO synthase (eNOS)−/− mice were infused with leptin (0.4 mg/kg per day, 7 days), and endothelium-dependent relaxation was studied in aortic segments. Leptin had no effect on acetylcholine-induced endothelium-dependent relaxation in normal wild-type mice but restored endothelium-dependent relaxation in wild-type mice treated with angiotensin II (0.7 mg/kg per day, 7 days) to induce endothelial dysfunction. Leptin also sensitized aortae from eNOS−/− mice to acetylcholine, an effect blocked by neuronal NOS (nNOS) inhibition and not observed in eNOS-nNOS double−/− mice. Consistent with these findings, leptin induced nNOS expression in murine and human vessels and human endothelial but not smooth muscle cells. Aortic nNOS expression was also induced in mice by a high-fat diet. Mechanistically, leptin increased endothelial Janus kinase 2 and signal transducer and activator of transcription 3 phosphorylation, and inhibition of Janus kinase 2 prevented nNOS induction in cultured cells and leptin-induced relaxations in eNOS−/− mice.

Conclusion—Leptin induces endothelial nNOS expression, which compensates, in part, for a lack of NO production by eNOS to maintain endothelium-dependent relaxation. (Arterioscler Thromb Vasc Biol. 2012;32:1605–1612.)

Key Words: leptin  ■  neuronal NO synthase  ■  vascular function

Depending on the nutritional state, adipose tissue contributes a variable but substantial part of the total body mass, and importantly affects whole-body metabolism, glucose, and energy homeostasis.1 A multitude of hormones, autacoids, and cytokines is produced in the adipose tissue with complex effects on the whole-body homeostasis. The stroma-vascular fraction of the adipose tissue is rich in inflammatory cells, and their number increases in proportion to the extent of obesity, making obesity a low-grade inflammatory condition.2

In addition to inflammatory cells, adipocytes themselves contribute to obesity-related signaling. Via adipokines, the hormones produced by adipocytes, information on the abundance and the activity state of the adipose tissue is communicated to the body with effects on food intake, energy expenditure, glucose and lipid metabolism, reproductive function, release of hormones from other tissues, and the vascular functional state.3 Although adipokines, in addition to tumor necrosis factor-α, are thought to mediate endothelial dysfunction in obesity, the hormone responsible for this effect has not yet been clearly identified.4

Appropriate blood supply by means of local control of blood flow and angiogenesis is prerequisite for adipose tissue expansion in obesity because these processes prevent adipocyte hypoxia and maintain nutrient supply to the fat.5 Numerous adipokines, including adiponectin, interleukin-6, apelin, and heptocyte growth factor contribute to this process.4 Among them, leptin was the first adipokine identified to promote angiogenesis.5,6 The hormone is a 16-kDa polypeptide produced by adipocytes in proportion to their triglyceride content. Leptin elicits anorexigenic responses in the brain, and positively affects whole-body energy expenditure, in part, by increasing sympathetic activity.6 The latter effect promotes thermogenesis in brown adipose tissue and thus energy expenditure but also increases the blood pressure.7,8 Via the leptin receptor and the subsequent activation of Janus kinase 2 (JAK2), leptin activates signal transducer and activator of transcription 3, AMP-activated protein kinase (AMPK), Akt, and extracellular signal-regulated kinase 1/2. These effects are considered saturable at a concentration of 20 ng/mL. Excessive concentrations of leptin, exceeding 50 ng/mL, have therefore been linked to leptin resistance.8

The leptin receptor is expressed in vascular as well as circulating cells, and thus leptin elicits numerous direct effects on the cardiovascular system. Nevertheless, it is still unclear...
whether these are rather beneficial or detrimental for cardiovascular function.14 Direct vascular effects of leptin have been studied in response to acute application in humans, animals, and isolated vessels with variable results depending on the experimental setting, species, and the concentration used. Whereas infusion of leptin increases the blood pressure,12 probably by stimulating the sympathetic nervous system,13 the hormone is also a direct vasodilator because after ganglion blockade, in vivo leptin lowered the blood pressure in rats.14,15 Although these observations have been questioned,16 organ chamber experiments of different vascular tissues of the rat using pharmacological concentrations of the hormone revealed that leptin elicits endothelium-dependent vasodilation by stimulating the release of NO or the endothelium-derived hyperpolarizing factor (EDHF).14,17 Leptin can activate the endothelial NO synthase (eNOS) by a calcium-independent mechanism involving Akt, AMPK, and eNOS serine 1177 phosphorylation.17 In contrast to this, the lepin-induced relaxation of human vessels was reported to be endothelium- and NO-independent.18,19 It should, however, be mentioned that many studies focusing on direct vasodilator effects of leptin were performed using supraphysiological concentrations of the hormone (usually 10 nmol/L which equals 160 ng/mL) whereas at physiological leptin concentrations (10 ng/mL) few responses were noted (as discussed in20).

Despite the considerable interest in leptin, few studies have focused on the vascular effects of prolonged in vivo infusion of physiological concentrations (≈10 ng/mL plasma concentration) of leptin. We herein report that prolonged administration of the hormone in mice results in the induction of neuronal NOS (nNOS). We demonstrate that in situations of high physiological leptin levels this process contributes, in part, to the maintenance of normal endothelial function in vascular disease models.

Materials and Methods

Endothelial function was determined by organ chamber experiments, and gene expression was analyzed with the aid of quantitative polymerase chain reaction. Plasma nitrite content was analyzed with the aid of the NO analyzer. Further details are available in the online-only Data Supplement.

Results

Leptin Improves Endothelium-Dependent Relaxation by an eNOS-Independent Mechanism

Plasma leptin levels were similar between wild-type (WT) and eNOS−/− mice and were increased ≈4-fold by leptin infusion (Figure 1A). In vivo leptin had little effect on the acetylcholine (ACh)-induced relaxation of aortic rings from WT mice but greatly improved responses in rings from eNOS−/− mice (Figure 1B). Relaxations to the potassium channel opener and endothelium-independent vasodilator cromakalim were not affected by in vivo leptin treatment (Figure 1C). The effect of leptin in eNOS−/− mice was mediated by a NO synthase because it was blocked by L-nitro-arginine methyl ester, a nonisoform-selective NOS inhibitor (Figure 1D). NOS-dependent vasodilatation in general is mediated by NO, but also hydrogen peroxide (H2O2) acting as an EDHF that can be released from NOS. Indeed, ex vivo catalase incubation attenuated the dilator response in aortic rings of leptin-treated eNOS−/− mice (Δ=34.6±6.1% of Emax, n=6, P<0.05). These data suggest that approximately a third of the L-nitro-arginine methyl ester-sensitive response of the vessels is mediated by H2O2 whereas the remaining portion should be a consequence of NO formation. Indeed, plasma nitrite measurements revealed that in WT mice in vivo leptin increased NO formation by ≈30% (Figure 1E). The increased NOS-dependent dilatation was also dependent on the presence of the endothelium, and its removal resulted in a complete loss of relaxation to ACh (remaining relaxation 3±3%, n=5). Because, however, smooth muscle cells of mice do not express muscarinic receptors, a second indirect approach was sought to exclude smooth
muscle as a source of nNOS. It has been demonstrated that an increase in vascular tension increases NO release from preconstricted vascular segments, and that the amount of NO released then counteracts the vasoconstriction.\(^{21}\) Indeed, in vivo leptin resulted in an attenuation of the phenylephrine-induced constrictor response, and this effect was not observed after the removal of the endothelium (Figure 1F).

**Leptin Improves Endothelium-Dependent Relaxation by Induction of nNOS Expression**

Due to the low expression level of nNOS and inducible NOS (iNOS) in murine vessels Western blotting could not be used. Rather, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to identify the NO synthase isoform induced in response to leptin. The technique revealed that in vivo leptin treatment results in an increase in nNOS but not eNOS or iNOS expression in aortic segments from WT as well as eNOS\(^{-/-}\) mice (Figure 2A). Accordingly, the leptin-induced increase in endothelium-dependent relaxation (EDR) of aortic segments from eNOS\(^{-/-}\) mice was blocked by 1-(2-trifluoromethylphenyl) imidazole, a NOS inhibitor, which had little effect on the dilator responses of WT aortic segments (Figure 2B). Most importantly, when leptin was infused into eNOS/nNOS double knockouts, it did not improve EDR (Figure 2C), clearly demonstrating that leptin-mediated induction of nNOS, in part, compensates for a lack of eNOS-derived NO production.

**Also in Human Cells, Leptin Induces nNOS**

With respect to its neuronal effects, mice are known to be significantly more sensitive to leptin than humans, and thus we wondered whether leptin-induced nNOS expression also occurs in human vessels. Indeed, ex vivo incubation of human arteries with leptin also resulted in enhanced nNOS expression (Figure 2D). As in murine vessels, this effect appears to be mediated by endothelial cells because leptin selectively increased nNOS expression in human umbilical vein endothelial cells (HUVECs) whereas human aortic smooth muscle cells did not respond (Figure 2E and 2F).

**JAK2/STAT3 Pathway Mediates Leptin-Induced nNOS Expression**

To determine the mechanism of leptin-induced nNOS expression, pharmacological inhibitors were used. Leptin-mediated nNOS induction in ex vivo incubated aortic rings of WT mice was blocked by JAK2, p38 mitogen-activated protein kinase, and phosphatidylinositol 3-kinase inhibition but not by extracellular signal-regulated kinase 1/2 (Erk1/2) inhibition (Figure 3A). Indeed, in HUVECs, leptin induced a rapid increase in p38 mitogen-activated protein kinase and JAK2 phosphorylation as well as of the phosphorylation of the JAK2 target signal transducer and activator of transcription 3 (STAT3) (Figure 3B). We therefore determined...
Aortic nNOS Expression Is Increased in Obese Mice

Although leptin induced nNOS expression in WT animals, it had little effect on EDR. This is expected because vessels of young, healthy mice respond so well to ACh that it is hard to further improve this response. Thus, we aimed to identify whether JAK2 also contributes to leptin-induced improvement of EDRs in vivo. In vivo cotreatment of leptin-infused mice with the JAK2 inhibitor AG490 blocked nNOS mRNA induction (nNOS mRNA without leptin 1±0, with leptin 0.85±0.17, n=5, P=nonsignificant) and prevented the leptin-induced improvement of EDR in aortae from eNOS−/− mice (Figure 3C).

Discussion

In the present study, we provide evidence that leptin induces nNOS in the endothelium, and this effect may, in part, compensate for the lack of eNOS-dependent NO production. The system is also functional in human vessels and contributes to the maintenance of EDR in obese mice and in angiotensin II–induced endothelial dysfunction. Induction of nNOS resulted in an improvement of EDR in eNOS−/− mice. This response was completely sensitive to L-nitro-arginine methyl ester and ≈30% to catalase, suggesting the nNOS-derived NO production makes 70% of the effect of leptin on EDR whereas the rest was mediated by H2O2. Indeed, L-nitro-arginine methyl ester is known to block NOS-dependent H2O2 production of normal endothelial function.

In Vivo Leptin Counteracts Endothelial Dysfunction

Given the small beneficial effect of the hormone in vessels with normal endothelial function, the effect of in vivo leptin treatment in angiotensin II–induced endothelial dysfunction was studied. Rings from WT animals treated with angiotensin II exhibited attenuated endothelium-dependent responses, and this effect was completely prevented by coinfusion of leptin (Figure 5A). The effects of leptin and angiotensin II, however, showed some interaction which limits the interpretation of these results. In vivo angiotensin II treatment further increased the plasma leptin level in leptin-infused mice (Figure 5B). More importantly, although blood pressure was slightly increased by leptin infusion in the absence of angiotensin II, leptin significantly attenuated the hypertensive effect of angiotensin II (Figure 5C). Because hypertension and endothelial dysfunction coincide in many disease models and may mutually account for each other, the leptin-induced improvement of endothelial function could well be a consequence of the antihypertensive effect of the hormone in angiotensin II–infused mice. To overcome this interaction, aortic rings were cultured ex vivo for 24 hours in the presence or absence of leptin and angiotensin II. Importantly, the results were similar to the in vivo situation. The angiotensin II–induced attenuation of ACh-induced relaxation was prevented by cotreatment with leptin (Figure 5D). This effect also appeared to be mediated by nNOS induction, because it was not observed when the nNOS inhibitor 1-(2-trifluoromethylphenyl) imidazole was present during the ACh-induced relaxation (Figure 5E).
formation, and H₂O₂ has been characterized as an nNOS-derived EDHF in eNOS−/− mice previously.

Because Western blotting of murine vascular tissue is not suited to detect nNOS expression, we used qRT-PCR to determine whether leptin-mediated induction of this enzyme is restricted to eNOS−/− mice. Interestingly, we observed that also in healthy mice and human vessels as well as human endothelial but not smooth muscle cells, leptin treatment results in nNOS induction. The tissue selectivity of this effect might be a consequence of the lower abundance of leptin receptors in the smooth muscle layer compared with endothelial layer. Importantly we used only a physiological concentration of 10 ng/mL leptin in the present study. Previous reports on the effect of leptin on smooth muscle cells used concentrations ≈20-fold higher than the one used here, which resulted in iNOS induction in this vascular layer.

Few studies have focused on nNOS as a mediator of the effect of leptin. In the brain, leptin maintains nNOS expression in the hypothalamus, and a high-fat diet increases cerebral nNOS expression. Cardiac nNOS expression is greatly attenuated in the leptin-deficient ob/ob mice and restored by leptin treatment. Thus, it cannot be excluded that vascular induction of nNOS so far was overlooked as a result of the traditional focus of the field on eNOS.

Expressed in the endothelium, nNOS, because of its calcium sensitivity, should resemble many characteristics of eNOS, and this is nicely demonstrated by the ability of nNOS to act as a generator of an EDHF in eNOS−/− mice. Obviously, eNOS−/− mice exhibit a greatly improved sensitivity to endothelium-dependent vasodilators, and thus a role of nNOS as mediator of EDR under physiological conditions is unlikely. Indeed, in normal mice, nNOS induction had little effect on EDR, and also previous studies reported that although leptin increases NO production in healthy mice, this has no impact on EDR.

Nevertheless, leptin treatment in ob/ob mice or in mice subjected to chronic intermittent hypoxia improved EDR. Also in the present study, we provide evidence that leptin contributes to the maintenance of vascular function in disease situations. We observed that a high-fat feeding protocol which does not result in massive diabetes mellitus or metabolic syndrome results in vascular nNOS induction, which coincided with increased plasma nitrite level, a marker for NO production. These data are in accordance with studies reporting that high-fat diet increases cerebral nNOS expression and cerebral nitrite content, and that plasma nitrite level in humans correlates with body fat mass. Importantly, in early diet-induced obesity in mice, NO production also of the adipose tissue is rather increased than decreased.
As a second model of endothelial dysfunction, we studied angiotensin II–induced hypertension. In vivo coinfusion of leptin and angiotensin II prevented the induction of endothelial dysfunction, and also ex vivo incubation of aortic rings with leptin blocked the endothelial dysfunction induced by angiotensin II. Importantly, we observed that the protective effect of leptin was not observed when EDR was studied in the presence of an nNOS inhibitor, suggesting that also in this particular model, nNOS induction contributes to the maintenance of EDR. An interaction of leptin and angiotensin II, different from angiotensin II, does not induce the NADPH oxidase. Moreover, when we determined the effect of leptin on the angiotensin II–induced H₂O₂ formation of angiotensin II type I receptor expressing HUVECs, we observed that leptin completely blocked the angiotensin II–induced reactive oxygen species formation (K. Schröder, 2012, unpublished observation), giving rise to the possibility that the beneficial effect of in vivo leptin on angiotensin II–induced hypertension and endothelial dysfunction are, in part, mediated by NADPH oxidase inhibition. Indeed, it was unexpected that leptin in the presence of angiotensin II lowered the blood pressure in mice. It is known that leptin increases the sympathetic tone, and also in the present study, we observed that in vivo leptin increased the blood pressure of control animals. Importantly, despite demonstrating increased sympathetic tone in mice in response to leptin, a recent study using telemetry failed to observe an effect of leptin on heart rate and blood pressure. As a result of the superior telemetry technique, mean heart rates were low in that study, and also the authors discuss that previous reports on a strong hypertensive effect of leptin might be, in part, consequence of high concentrations used, traumatic injection, or more stressful techniques of blood pressure recordings. Moreover, the direct impact of blood pressure on endothelial function is far from clear, and under certain model conditions, the ACh-induced relaxation even increases in the course of hypertension development.

Angiotensin II increased plasma leptin levels, which is in line with previous reports from cultured adipocytes but contrasts a previous observation in rats. Unexpectedly, also the high plasma levels in mice receiving leptin infusion were further increased by angiotensin II. This raises the possibility that angiotensin II not only impacts on leptin production but also leptin clearance. Indeed, leptin is removed from the body by renal excretion, and the fall in glomerular filtration rate in response to angiotensin II infusion could result in leptin accumulation.

In conclusion, we focused on the responses to prolonged in vivo treatment of low, physiological doses of leptin in mice. We observed that the hormone induced nNOS expression, and this contributed to EDR in aortic segments of healthy eNOS−/− and in C57BL/6 mice after angiotensin II treatment. Leptin increased the plasma levels of nitrite, a marker for NO vessels, however, a different mechanism appears to be operative, and our data suggest nNOS induction to be of relevance. In addition to this, leptin may also interfere with the vascular redox balance. Acute leptin signaling requires a transient activation of the NADPH oxidase NOX2, and thus an acute challenge with leptin produces a transient increase in reactive oxygen species formation, which is comparable with that observed in response to other protective hormones like hepatocyte growth factor or erythropoietin and involved in the physiological signaling of the hormone. This effect, however, appears to be lost after prolonged treatment, and in ob/ob mice, leptin reduces markers of oxidative stress. Moreover, although a correlation between the plasma level of leptin and NOX activity in human monocytes was reported, in an experimental model, leptin, different from angiotensin II, does not induce the NADPH oxidase. Moreover, when we determined the effect of leptin on the angiotensin II–induced H₂O₂ formation of angiotensin II type I receptor expressing HUVECs, we observed that leptin completely blocked the angiotensin II–induced reactive oxygen species formation (K. Schröder, 2012, unpublished observation), giving rise to the possibility that the beneficial effect of in vivo leptin on angiotensin II–induced hypertension and endothelial dysfunction are, in part, mediated by NADPH oxidase inhibition. Indeed, it was unexpected that leptin in the presence of angiotensin II lowered the blood pressure in mice. It is known that leptin increases the sympathetic tone, and also in the present study, we observed that in vivo leptin increased the blood pressure of control animals. Importantly, despite demonstrating increased sympathetic tone in mice in response to leptin, a recent study using telemetry failed to observe an effect of leptin on heart rate and blood pressure. As a result of the superior telemetry technique, mean heart rates were low in that study, and also the authors discuss that previous reports on a strong hypertensive effect of leptin might be, in part, consequence of high concentrations used, traumatic injection, or more stressful techniques of blood pressure recordings. Moreover, the direct impact of blood pressure on endothelial function is far from clear, and under certain model conditions, the ACh-induced relaxation even increases in the course of hypertension development.

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production, and nNOS induction was not only observed in human vessels in response to leptin treatment but also in mice subjected to high-fat diet. Thus, nNOS is a novel mediator of the beneficial effects of leptin.

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Disclosures
None.

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Leptin potentiates endothelium-dependent relaxation by inducing endothelial expression of neuronal nitric oxidase synthase (nNOS)


Study Design and Animal Procedures

eNOS knockout mice were bred at the local facility, eNOS-nNOS double knockout mice were provided by one of the co-authors. C57Bl6 control mice were obtained from Charles River Laboratories, Sülzfeld, Germany. At the age of 8 weeks, animals were subjected to sham or leptin infusion by osmotic minipumps (0.4 mg/kg/d). In subgroups AG490 was injected daily (2 mg/kg/d, i.p.) or angiotensin II (0.7 mg/kg/d) was co-administered by the osmotic pump. Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local government. Male wild-type mice (4 weeks old) were fed a normal chow or a high fat diet containing 35% lard (Teklad diet TD.03584, Harlan Laboratories Inc, Madison, WI, USA) for 8 months. Body weight and fasting plasma glucose were monitored monthly. To determine glucose tolerance, mice were starved for 16 hours after which they received 1 mg/g body weight glucose (as a 25% solution in water) by oral gavage. Plasma glucose was measured directly before and 15, 30, 60 and 120 minutes after glucose administration in venous blood from a small tail clip.

Organ Culture

Murine aortic segments or human vascular segments obtained during operations were dissected under sterile conditions, cleaned, and incubated for 16 hours at 5% CO2 and 37°C in MCDB 131 culture medium (Gibco) containing 0.1% BSA, penicillin, and streptomycin in the presence or absence of leptin (10 ng/ml) with or without inhibitors. In some cases endothelium was removed by a short treatment with CHAPS (5 mg/mL dissolved in glucose solution 50g/L, exposure for 45 seconds). Subsequently, the tissue was snap frozen in liquid nitrogen and mRNA was extracted for quantitative RT-PCR. The use of human tissue, otherwise discarded during operations, was approved by the local ethics committee.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords by dispase digestion or obtained from Lonza (Basel, Switzerland). HUVECs were used between passages 1-3 and cultured in EBM-medium supplemented with 10% FCS and bovine brain supplement. Human aortic smooth muscle cells were obtained from Lonza, used between passages 3-6 and cultured in EBM-medium supplemented with 10% FCS, bovine brain supplement and human recombinant insulin.

Immunoblotting

Western blot analyses were performed with an infrared-based detection system (Odyssey, Licor, Bad Homburg, Germany). All primary antibodies were purchased from Cell signaling and infrared-fluorescent-dye-conjugated secondary antibodies were obtained from Licor (Bad Homburg). The following lysis buffer was used (pH 7.4, concentrations in mmol/L): Tris-HCl (50), NaCl (150), sodium pyrophosphate (10), sodium fluoride (20), nonidet P40 (1%), sodium deoxycholate (0.5%), proteinase inhibitor mix, phenylmethylsulfonyl fluoride (1), orthovanadate (2), okadaic acid (0.00001).

Real-Time PCR
Primers against NOS isoforms were designed using the sequence information of the NCBI database (5′→3′): hum NOS3 (eNOS) fw CTG CCC TGG CCG AGG AGA CT; rev CAG CAC GTC GAA GCG ACC GT; hum NOS2 (iNOS) fw GAC CTG GGA CCC GCA CCA CT; rev AGG ATG GTG GCA CGG CTG GA; hum NOS1 (nNOS) fw CAT CAG GCG ATG GGC CCG AC; rev AGC GTG TCC GAA GCC CCA AA; hum leptin receptor fw GGT CCA GCC CAC CAT TGG TAC C; rev GGC CAT CCA GTC TCT TGC CCC T; murine leptin receptor fw CGG GAC ACA GGT GGG ACA CTC TTT; rev CAA CCC ACC CTC TTT CCG CCT C mur. Nos1 (nNOS) fw CTC CCG CCT CGG GCA AAC AG; rev GTG CAC CCC GTT CAC AGC GT; mur. Nos2/iNOS fw GCT CGC TTT GCC ACG GAC GA; rev AAG GCA GCG GGC ACA TGC AA; mur. NOS3 (eNOS) fw CTC ACC ATA GCT GTG CTG TAC; rev GAT GCA GGG CAA GTT AGG ATC AGG. Total RNA was isolated from aortic rings and cells with the "Absolutely RNA RT PCR Miniprep kit" (Stratagene Europe), and used for reverse transcription (Superscript III RT, Invitrogen) with pdN6 primers (Amersham/Pharmacia). The polymerase chain reaction (PCR) conditions were as follows: initial denaturation, 95°C, 10 minutes; 40 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 60 seconds), and elongation (72°C, 60 seconds).

Statistics

All values are mean±SEM. Relaxations were calculated from individual dose-response curves. Statistical analysis was carried out by ANOVA for repeated measurements or determination of the area under the curve followed by Fisher's least significant difference test or paired t test, if appropriate. Values of p<0.05 were considered statistically significant.

Reference List