Absence of Cardiotrophin 1 Is Associated With Decreased Age-Dependent Arterial Stiffness and Increased Longevity in Mice

Natalia López-Andrés, Laurent Calvier, Carlos Labat, Renaud Fay, Javier Díez, Athanase Benetos, Faiez Zannad, Patrick Lacolley, Patrick Rossignol

Abstract—Cardiotrophin 1 (CT-1), an interleukin 6 family member, promotes fibrosis and arterial stiffness. We hypothesized that the absence of CT-1 influences arterial fibrosis and stiffness, senescence, and life span. In senescent 29-month-old mice, vascular function was analyzed by echotracking device. Arterial histomorphology, senescence, metabolic, inflammatory, and oxidative stress parameters were measured by immunohistochemistry, reverse transcription polymerase chain reaction, Western blot, and ELISA. Survival rate of wild-type and CT-1–null mice was studied. Vascular smooth muscle cells were treated with CT-1 (10^{-9} \text{ mol/L}) for 15 days to analyze senescence. The wall stress-incremental elastic modulus curve of old CT-1–null mice was shifted rightward as compared with wild-type mice, indicating decreased arterial stiffness. Media thickness and wall fibrosis were lower in CT-1–null mice. CT-1–null mice showed decreased levels of inflammatory, apoptotic, and senescence pathways, whereas telomere-linked proteins, DNA repair proteins, and antioxidant enzyme activities were increased. CT-1–null mice displayed a 5-month increased median longevity compared with wild-type mice. In vascular smooth muscle cells, chronic CT-1 stimulation upregulated apoptotic and senescence markers and downregulated telomere-linked proteins. The absence of CT-1 is associated with decreased arterial stiffness, fibrosis, and senescence and increased longevity in mice likely through downregulating apoptotic, senescence, and inflammatory pathways. CT-1 may be a major regulator of arterial stiffness with a major impact on the aging process.  

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Key Words: vascular stiffness ■ aging ■ fibrosis ■ cytokines

Cardiotrophin 1 (CT-1) is a member of the interleukin 6 superfamily that interacts with the heterodimer constituted by the glycoprotein 130 and the leukemia inhibitory factor receptor-β. Our group has described that CT-1 induces cell proliferation, hypertrophy, and secretion of extracellular matrix proteins in aortic vascular smooth muscle cells (VSMCs) in vitro and in vivo, suggesting a role for CT-1 in arterial fibrosis and stiffness, which both are features of aging. Furthermore, elevated plasma concentrations of CT-1 have been reported in patients with a variety of diseases, including hypertension, heart failure, unstable angina, myocardial infarction, and aortic stenosis, indicating that this cytokine may contribute to the development of cardiovascular pathologies associated with aging.

Arterial walls stiffen with age. The arterial wall is a complex tissue composed of different cell types subject to structural and functional modifications. Age-associated changes in blood vessels include the increase in the inflammatory response, the cell loss, the inability to repair DNA damage, the oncogenic activation, and the regulation of telomere-telomerase complex. A number of age-associated structural, functional, and molecular changes occur in the arterial system. Aging is associated with thickening and dilatation of large arteries, extracellular matrix accumulation, calcium deposits, increased vascular stiffness, and endothelial dysfunction. It has been suggested that these alterations are attributable to age-related functional changes in vascular cells. The age-associated arterial inflammatory phenotype includes increased expression of monocyte chemoattractant protein 1, intercellular adhesion molecule 1, matrix metalloproteinase-2 activity, or transforming growth factor-β1 expression.

Here, we investigated whether the absence of CT-1 in mice confers protection from arterial stiffness and fibrosis with an impact on vascular senescence and overall mortality. To understand the effects of CT-1 in arterial stiffness associated with aging, we studied a cohort of senescent (29-month-old) CT-1–null mice and their wild-type (WT) littermates.
Vascular function, inflammation, senescence, telomere-linked proteins, DNA repair proteins, antioxidant enzyme activities, and mortality were measured. Moreover, primary VSMCs were used to further characterize the effect of CT-1 chronic treatment on senescence in vitro.

### Methods

#### Animals and Assessment of Life Span

The investigation was performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. WT and CT-1–null mice backcrossed into a C57BL/6 background were obtained from D. Pennica (Genentech). Animals were housed in a constant temperature room, with a 12-hour dark/12-hour light cycle, and allowed free access to standard diet and water. Only male littermates were used. A number of mice were euthanized at the age of 22 (4 WT and 7 CT-1–null), 29 (14 WT and 13 CT-1–null), and 36 months (4 CT-1–null). For functional, histological, molecular, and biochemical studies (heart, aorta, and blood), only 29-month-old WT (n=14) and CT-1–null (n=13) mice were used.

#### Biochemical Measurements

Blood glucose levels were measured using a Glucometer Elite (Bayer) at month 29, after overnight fasting. Plasma insulin, leptin, and resistin levels were determined with ELISAs (Linco Research). Plasma CT-1, C-reactive protein, plasminogen activator inhibitor 1, tumor necrosis factor-α, and interleukin 4 levels were measured by ELISA (R&D Systems). The activity of superoxide dismutase and glutathione in plasma was quantified by ELISA (Cayman Chemical).

#### In Vivo Carotid Mechanical Properties

We recorded intra-arterial diameter of the carotid artery and blood pressure (BP) in isoflurane-anesthetized old mice using an ultrasonic echotracking device (NIUS-01, Asulab SA), as described previously.16

#### Morphological and Histological Evaluation

Histological determinations in vascular tissue were performed in 5-μm–thick sections stained with hematoxylin for carotid morphology, with orcein for elastin content and with Sirius red for collagen content, as reported previously.17 Media thickness has been measured in triplicate in a minimum of 10 WT and 10 CT-1–null mice. For the identification of DNA fragmentation, terminal deoxyribonucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was performed following the manufacturer’s instructions (Roche).

#### Reverse Transcription and Real-Time Polymerase Chain Reaction

Total RNA extraction was performed using a nucleic acid purification lysis solution (Applied Biosystems) and the semiautomated ABI PRISM 6100 Nucleic Acid PrepStation system (Applied Biosystems). Real-time polymerase chain reaction was performed with an ABI PRISM 7000 Sequence Detection System by using specific TaqMan MGB fluorescent probes (Applied Biosystems). Constitutive 18S ribosomal RNA was used as endogenous control.

#### Western Blot

Aortas and VSMCs were homogenized in lysis buffer (Roche). Protein concentration was determined by the Lowry method (BioRad). Proteins (30 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Biosciences). Blots were visualized using an ECL-Plus chemiluminescence detection system (Amersham Biosciences). Bands were quantified by densitometry and normalized by β-actin values.

#### Cell Cultures

Primary rat aortic VSMCs were isolated by using standard enzymatic dissociation techniques.2 Cells were plated and grown in DMEM (Invitrogen) supplemented with 5% FBS and cultured at 37°C in air.
containing 5% CO₂. Cells were used at passages 2 to 6. Before treatments, cells were serum starved for 12 hours. In all of the experiments, to expose to CT-1 (10⁻⁹ mol/L) for the indicated times, the conditioned medium was aspirated, and we added quickly the fresh culture medium without FBS every 24 hours.

**Senescence-Associated β-Galactosidase Staining**

Senescence-associated β-galactosidase (SA-β-gal) activity was detected as a biomarker for cellular senescence. Cell that were 50% to 60% confluent and quiescent cultured on Lab Tek chambered coverglass (Nunc) were exposed to CT-1 (10⁻⁹ mol/L) for 1, 7, and 15 days. SA-β-gal staining was performed using a Senescence Detection kit (Sigma). The number of total and SA-β-gal-positive cells was counted in 10 randomly chosen fields per group at ×20 magnification.

**Statistical Analysis**

Results are presented as mean±SE, computed from the average measurements obtained from each group of animals or cells. The time occurrence of death (months) was assessed by Kaplan-Meier cumulative survival functions. Survival distribution between the 2 groups of mice was compared using the log-rank test. Normal distribution of data was checked using the Shapiro-Wilk test. A Levene statistic test was performed to check the homogeneity of variances. Differences among more than 2 experimental conditions were tested by the ANOVA 1-way test, followed by the Scheffe test to analyze differences between groups. The unpaired Student t test or the Mann Whitney U test was used to assess statistical differences between 2 experimental conditions. P values <0.05 were considered significant.

**Results**

**Absence of CT-1 Is Associated With Decreased Arterial Stiffness**

The heart weight, the heart weight:body weight ratio, the kidney weight, and the kidney weight:body weight ratio were comparable between CT-1–null and WT mice. BP and heart rate were slightly increased in mice lacking CT-1 (Table). There were no changes in carotid diameter, compliance, and distensibility calculated at mean BP. By contrast, increment elastic modulus (Eₘₑ) and wall stress (WS) values were higher (P<0.01) in CT-1–null mice when compared with WT animals. The diameter-arterial pressure curve in CT-1–null mice tended to be shifted upward when compared with the curve for WT animals (Figure 1A). The distensibility-arterial pressure curve in CT-1–null group was shifted upward (P<0.05) compared with the WT group, indicating that mice lacking CT-1 presented higher distensibility for a given BP (Figure 1B). As for the Eₘₑ–WS curves, they shifted to the right (P<0.05), toward higher values of WS (in comparison with WT mice) (Figure 1C). The comparison between WT and CT-1–null mice curves was made by calculating the mean WS within the 350- to 1000-kPa range of Eₘₑ (MWS₃₅₀–₁₀₀₀). In CT-1–null mice, MWS was increased (P<0.01), indicating a decrease in stiffness (Figure 1D). Thus, the absence of CT-1 was associated with increased arterial distensibility and decreased stiffness of the arterial wall material.

**Absence of CT-1 Is Associated With Decreased Arterial Fibrosis**

As can be seen in the Table, carotid media cross-sectional area and media thickness were lower in CT-1–null animals when compared with WT mice (20% and 33%, respectively; representative photographs are shown in Figure 1E). Moreover, CT-1–null mice had a lower carotid collagen density (40%; P<0.01) than WT mice, but the absence of CT-1 did not affect elastin amounts (Figure 1E). In accordance with these observations, the aortic media cross-sectional area and the aortic media thickness were also diminished in CT-1–null mice when compared with WT. These differences were not because of VSMC proliferation, because the percentage of ki-67-positive nuclei was similar in WT and CT-1–null mice (Table). The aortic expression of α₁-procollagen mRNA was reduced (40%; P<0.01), as was the expression of collagen type I (60%; P<0.01) and collagen type III (35%; P<0.01) in CT-1–null mice compared with WT mice (Figure 1F). Fibronectin mRNA and protein expression were also decreased in aorta from mice lacking CT-1 compared with WT mice (30% and 45%; P<0.01, respectively; Figure 1F). Furthermore, matrix metalloproteinase-2, -9, and -13 activities were reduced (55%, 45%; P<0.01) in mice lacking CT-1, whereas Bcl-2 expression was increased (64%; P<0.01). Moreover, old CT-1–null mice exhibited decreased cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (47% and 40%, respectively; P<0.05), when compared with WT mice. These results were confirmed by TUNEL and poly (ADP-ribose) polymerase staining (Figure 2B), showing low apoptosis in aorta from mice lacking CT-1. This decreased aortic apoptosis in CT-1–null mice, but not in WT animals, was accompanied by a decrease in the expression of senescence markers. As shown in Figure 2C, vascular p53 was diminished in mice lacking CT-1 (38%; P<0.05). The expression of Mdm2, the principal cellular antagonist of p53, was enhanced (88%; P<0.01) in old CT-1–null animals when compared with WT. The expression of the others senescence markers, p21 and p16, was also decreased in aortas from CT-1–null mice when compared with WT (44% and 45%, P<0.05, respectively).

To analyze whether the absence of CT-1 could alter the proteins that protect telomeres, vascular expression of DNA-dependent protein kinase catalytic subunit, ku86 (the regulatory component of the DNA-associated protein kinase), TRF-2 (telomeric repeat-binding factor-2 that regulate telomerase by protecting the chromosome ends), and TERT (catalytic subunit of the enzyme telomerase) was quantified. As shown in Figure 2D, the expression of all of these proteins was augmented in aorta from CT-1–null mice when compared with WT (90%, 300%, 300%, and 450%, P<0.01, respectively).

Figure 2E shows vascular expression of nuclear factor-κB (NFκB) pathway, which regulates cellular responses and is the first responder to harmful cellular stimuli, in WT and
CT-1–null mice. Mice lacking CT-1 presented decreased vascular phosphorylation of IKK (IκB kinase) α/β (46%, P<0.01), IxBα (30%, P<0.05), and NFκB (47%, P<0.01) when compared with WT mice.

Absence of CT-1 Is Associated With Increased Antioxidant and Decreased Inflammatory State

The analysis of the antioxidant system in 29-month-old CT-1–null mice showed an increased superoxide dismutase activity (200%, P<0.01) and glutathione activity (152%, P<0.05) when compared with age-matched WT controls (Table). Furthermore, mice lacking CT-1 presented decreased levels of plasma pro-inflammatory molecules C-reactive protein (48%, p<0.01), plasminogen activator inhibitor 1 (63%, P<0.01), and tumor necrosis factor-α (51%, P<0.01) and similar levels of the anti-inflammatory cytokine IL-4 when compared with WT mice (Table). In parallel, we investigated metabolic features in WT and CT-1–null mice. Morphological examination
Figure 2. Apoptotic, senescence markers, telomere-related proteins, and nuclear factor-κB (NF-κB) pathway activity in aorta from wild-type (WT) and cardiotrophin 1 (CT-1)–null mice. A, The apoptotic markers Bax, Bcl-2, caspase-3, and poly (ADP-ribose) polymerase (PARP) were evaluated in the aorta from old WT and CT-1–null mice. Old CT-1–null mice showed decreased vascular apoptosis when compared with WT. B, Representative microphotographs of TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) and PARP staining in aorta from WT and CT-1–null mice showed decreased apoptosis in CT-1–null mice when compared with WT. C, The senescence markers p53, Mdm2, p21, and p16 were evaluated in the aorta from old WT and CT-1–null mice. Old CT-1–null mice presented diminished vascular senescence when compared with WT. D, The telomere integrity markers DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ku86, TRF-2 (telomeric repeat-binding factor-2), and TERT (catalytic subunit of the enzyme telomerase) were evaluated in the aorta from old WT and CT-1–null mice. Old CT-1–null mice showed increased telomere-related proteins when compared with WT. E, Old CT-1–null mice presented diminished vascular NF-κB pathway activity when compared with WT. The images are representative of 3 independent experiments and histograms with bars represent the means±SE of ≤10 WT and 10 CT-1–null mice. *P<0.01 vs WT; †P<0.05 vs WT.
of 29-month-old mice revealed that CT-1–null mice presented an increase in body weight (12%, P<0.01) when compared with their littermates WT. Concentrations of fasting blood glucose and resistin did not significantly differ in CT and CT-1–null mice, whereas adiponectin levels were increased (130%, P<0.05) and leptin concentration was decreased (51%, P<0.05) in CT-1–null mice when compared with WT (Table).

Absence of CT-1 Is Associated With Increased Life Span in Mice
Among the 42 mice of the study, 18 (7 WT and 11 CT-1-null) were euthanized around 29 months for histological assessment: they were coded as censored at the time of euthanize in the survival analysis. The 4 surviving CT-1–null mice were euthanized at the end of the study (36 months). Twenty mice (11 WT and 9 CT-1-null) died spontaneously during follow-up. CT-1–null mice substantially outlived their WT littermates, with median survival time of 33 and 28 months, respectively (P=0.007) (Figure 3). There was no difference in life span between 1 and 21 months.

CT-1 Induces Apoptosis and Premature Senescence In Vitro in VSMCs
To determine whether CT-1 induced vascular apoptosis and senescence in vitro, VSMCs were treated with CT-1 for 1, 7, and 15 days. The percentage of SA-β-galactosidase-positive VSMC was significantly increased (P<0.01) by CT-1 in a time-dependent manner, starting 7 days after CT-1 stimulation (Figure 4A). CT-1 treatment also increased time-dependently (P<0.01) VSMC apoptosis assessed by Bax/Bcl-2 ratio, caspase-3 cleavage and Poly (ADP-ribose) polymerase cleavage time-dependently (Figure 4B), and the senescence markers p53, p21, and p16, without modifying Mdm2 expression (Figure 4C and 4D).

CT-1 Decreases Telomere Integrity and Telomerase Activity and Increases NFκB Pathway Activity In Vitro in VSMCs
The effects of CT-1 on the expression of proteins that protect telomeres were analyzed. As shown in Figure 5A and 5B, VSMCs treated with CT-1 for 15 days exhibited reduced levels of DNA-dependent protein kinase catalytic subunit, ku86, and TRF-2 expression (P<0.05). However, in both 15 days control and CT-1–treated cells, an increase (P<0.05) in TERT expression was found (Figure 5B). Moreover, CT-1 treatment increased (P<0.05) phosphorylation of IkBα and NFκB without modifying IKK (IκB kinase) α/β activation in VSMCs.

Discussion
In the present study, for the first time, we report that the absence of CT-1 translated into a marked reduction in arterial stiffness associated with aging. Mice lacking CT-1 developed less vascular fibrosis with lower deposition of extracellular matrix proteins, mainly collagen type I and fibronectin. This occurred despite slightly higher mean BP, however still in the reference range. This was associated with a lower inflammation and oxidative stress and a reduced senescence, resulting in a prolongation of the mice life span. Such phenomena are in line with our in vitro results showing that CT-1 treatment prematurely provoked cellular senescence in VSMCs.

Age-associated changes in blood vessels include a decrease in compliance, an increase in arterial stiffness and arterial wall thickening as a result of increased vascular calcifications, increased collagen content and cross-linking, and decreased elastin content. Thus, several clinical studies have shown a strong association between arterial stiffness and cardiovascular morbidity and mortality. In this regard, it has been described that CT-1 stimulated bone formation in vitro and in vivo and enhanced collagen synthesis and matrix metalloproteinase activities in VSMCs without modifying elastin levels, suggesting a role for the cytokine in vascular calcifications and arterial stiffness. According to these findings, aged mice lacking CT-1 presented decreased arterial stiffness and media thickness accompanied by diminished collagen and fibronectin content and decreased matrix metalloproteinase activities. In addition to structural changes, arterial stiffness is strongly affected by VSMC tone, which can be modified by mechanostimulation itself and by paracrine mediators, such as oxidative stress. Oxidative stress is a major contributor to aging. The observation of increased superoxide dismutase and glutathione activities in old mice lacking CT-1 compared with WT mice suggests that CT-1 may affect organ senescence though induction of oxidative stress. In this respect, it has been evidenced that CT-1 increased reactive oxygen species in cardiomyocytes, regulating Jak/Stat and NFκB signaling pathway, raising the possibility that CT-1 exerts its biological effects via an elevation of oxidative stress. In the present study, on stimulation of VSMCs with CT-1, Stat-3 phosphorylation, and NFκB signaling pathway activity increased. According to this, NFκB signaling pathway activity was not modified with aging in mice lacking CT-1, whereas the activity augmented with aging in WT mice. Thus, the absence of CT-1 protected mice from the deleterious process of oxidative stress maybe by downregulating NFκB signaling pathway activity.

Cellular senescence is characterized by biochemical events that occur within the cell leading to growth arrest and loss of specialized cellular functions. Senescent cells cannot...
Figure 4. Apoptotic and senescence markers in vascular smooth muscle cells (VSMCs) treated with cardiotrophin 1 (CT-1). A, CT-1 treatment enhanced SA-β-Gal activity. B, The apoptotic markers Bax, Bcl-2, caspase-3, and poly (ADP-ribose) polymerase (PARP) were increased by chronic CT-1 treatment. C and D, CT-1 enhanced the expression of the senescence markers p53, Mdm2, p21, and p16. The images are representative of 3 independent experiments and histograms with bars represent the mean±SE of ≤3 separate experiments. *P<0.05 vs control 1 day; $P<0.05 vs control 15 days.
Figure 5. Telomere-related proteins and nuclear factor-κB (NFκB) pathway activity in vascular smooth muscle cells (VSMCs) treated with cardiotrophin 1 (CT-1). A and B. The telomere integrity markers DNA-PKcs, ku86, TRF-2 (telomeric repeat-binding factor-2), and TERT (catalytic subunit of the enzyme telomerase) were evaluated in VSMCs treated with CT-1. Cells treated chronically with CT-1 presented decreased telomere-related proteins. C, NFκB pathway activity was enhanced on treatment with CT-1. D, CT-1 decreased Akt phosphorylation and increased Stat-3 phosphorylation in VSMCs. The images are representative of 3 independent experiments and histograms with bars represent the mean±SE of ≤3 separate experiments. *P<0.05 vs control 1 day; $P<0.05 vs control 15 days.
divide, their ability to synthesize proteins is reduced, and the DNA repair system is attenuated.\textsuperscript{26} CT-1–null mice did not exhibit differences in VSMC proliferation but presented reduced cardiovascular apoptosis and senescence than WT mice. Moreover, the expression of proteins implicated in DNA repair and telomere integrity decreased or remained constant with age in WT but increased in CT-1–null mice. All these data point at the protective effect of the absence of CT-1.

Another group has also looked at cohorts of WT and CT-1–null mice\textsuperscript{27} and found opposite trends regarding differences in leptin concentrations between the groups (12-month-old CT-1–null mice presented increased body weight and leptin levels). One potential explanation for the difference is that they studied a younger cohort (12-month-old of mice, because our result in old mice showed a more modest increase in body weight accompanied by decreased leptin and increased adiponectin levels, suggesting that aging CT-1–null mice did not exhibit features of metabolic syndrome. Moreover, although BP values in CT-1–null mice were slightly increased, they were in the reference ranges. In addition, we and others have previously published that chronic CT-1 treatment could exert harmful effects at the cardiovascular and metabolic levels. Thus, CT-1 impairs myocardial contractility,\textsuperscript{28} induces myocardial remodeling\textsuperscript{17} and inflammation,\textsuperscript{29} and induces the development of insulin resistance in vitro.\textsuperscript{30} The potential detrimental metabolic actions of CT-1 are supported by studies performed in patients with the metabolic syndrome in which an excess of this cytokine at the adipose tissue level and in the circulation were reported.\textsuperscript{31} Furthermore, circulating CT-1 was associated with glucose levels in these patients.\textsuperscript{31} These data should be considered together and more complex and integrative specific analyses should be performed to better understand the role of CT-1 treatment in metabolic disturbances.

**Perspectives**

Our findings have provided evidence that in the absence of CT-1 arterial stiffness is reduced in association with consistent patterns of diminished apoptosis, senescence, inflammation, and oxidative stress, potentially contributing to increased longevity. We thus suggest that CT-1 emerges as a target candidate to interfere with arterial stiffness associated to the aging process.

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**Disclosures**

None.

**References**


What Is New?
• The absence of cardiotrophin 1 (CT-1) is associated with reduced arterial fibrosis and stiffness and delayed arterial senescence. Thus, CT-1 may be a major regulator of arterial stiffness with a major impact on the aging process.

What Is Relevant?
• Our results are particularly original, because antistiffening strategies (beyond antihypertensive treatment) are not available so far and are therefore an important unmet clinical need. CT-1 could be a new therapeutic target to decrease arterial stiffness associated with the aging process.

Summary
The absence of CT-1 is associated with decreased arterial fibrosis, stiffness, and senescence and increased longevity in mice likely through downregulating apoptotic, senescence, and inflammatory pathways. CT-1 may be a major regulator of arterial stiffness with a major impact on the aging process.
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