The effect of losartan *in vitro* and *in vivo* on muscle signalling and function and antisense oligonucleotide-mediated exon skipping

Chapter 7

Introduction

The renin-angiotensin system (RAS) plays a role in the maintenance of blood pressure and fluid balances and is, amongst others, involved in the development of cardiomyopathy. Upon activation, renin is secreted by the kidneys, which converts angiotensinogen, an α-glycoprotein released by the liver, into angiotensin I. Angiotensin I in turn is converted in its active form angiotensin II by the membrane-bound metalloproteinase angiotensin converting enzyme (ACE), which has numerous effects, amongst others on vascular smooth muscle cells to induce vasoconstriction. It exerts its main effects by binding to the angiotensin II type 1 (AT1) receptor, but also binds to angiotensin II receptor type 2 (AT2).[714] This system has shown to be upregulated in DMD and increases fibrosis by both transforming growth factor-β (TGF-β)-dependent and independent mechanisms.[554,555]

TGF-β is a member of the TGF-β superfamily, proteins that are involved in the control of many cellular processes such as growth and differentiation. TGF-β mainly signals via the type II receptor Tgfbr2 in combination with the type I receptor Tgfbr1 (ALK5). TGF-β_1, one of the three mammalian isoforms, is known to be induced in regenerating muscle and is a potent inducer of fibrosis. Downstream signalling occurs via the canonical Smad2/3-dependent pathway and non-canonical Smad-independent pathways.[478] TGF-β_1 is upregulated in patients and mice lacking dystrophin, a protein that is crucial to maintain muscle fibre stability during contraction and lack of which leads to severe muscle damage and replacement of muscle tissue by fibrotic and adipose tissue. The level of TGF-β_1 expression has shown to be correlated with disease severity in dystrophin negative Duchenne muscular dystrophy (DMD) patients and *mdx* mice.[715,716]

AT1 stimulates TGF-β via the NADPH oxidase/P38/MAPK pathway, thereby enhancing downstream signalling.[558] TGF-β-independently AT1 activation works directly on the PI3K/Akt/mTOR and Raf/MEK/ERK pathways, which also induce fibrosis.[561,562]

Losartan is a selective AT1 receptor antagonist, thereby anticipated to have an antagonistic effect on TGF-β. In *mdx* mice it improved regeneration and reduced fibrosis after acute injury. Six to nine months of treatment ameliorated the disease progression evidenced by a decrease in fibrosis and improving muscle strength.[260]

Antisense oligonucleotide (AON)-mediated exon skipping, described in more detail in previous chapters, targets *DMD* transcripts, which are only expressed in muscle fibres and not in fibrotic/adipose tissue that gradually replaces the muscle fibres when the disease progresses. Indeed the first clinical trial in DMD patients using local intramuscular injection of AONs targeting exon 51 already showed that patients with better muscle quality were able to produce more dystrophin.[225] Therefore combining AON-treatment with a muscle preserving agent might increase the therapeutic effects of AON-mediated exon skipping. In light of the described effects of losartan for preserving muscle quality, in this chapter the effects of losartan itself and in combination with AONs were examined.

Materials and methods

Cell culture

A mouse myoblast cell line (C2C12) and primary human myoblasts derived from a healthy control (KM109)[697] and a DMD patient with a deletion of exon 51-55 (DL589.2)[177] were used in this study.

Mouse C2C12 myoblasts were cultured on a collagen layer (1:30; Pure Col; Nutacon

BV; the Netherlands)at 37ºC 10% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, without phenol red; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin streptomycin (P/S), 2% GlutaMAX and 1% D-glucose (all from Gibco-BRL, Life Technologies; the Netherlands).

Human myoblast were cultured on a collagen layer (1:30; Pure Col; Nutacon BV) in Nutrient Mix F-10 (HAM) supplemented with GlutaMAX (Gibco-BRL), 20% FBS and 1% P/S at 37ºC 5% CO_2. To induce differentiation into myotubes medium was switched into DMEM without phenol red supplemented with 2% FBS, 1% P/S, 2% GlutaMAX and 1% D-glucose (Gibco-BRL) when cells reached 80-90% confluence.

Smad phosphorylation assay

For the Smad phosphorylation assay C2C12 cells were seeded in a 6-well plate at a density of $3 \cdot 10^5$ cells/well. After overnight serum starvation, cells were treated for 1 hour with 0, 0.5 or 50 µg/mL losartan potassium (Cozaar®; Merck Sharp & Dohme BV; the Netherlands) or 10 µM Transforming Growth Factor-β type I receptor kinase inhibitor (LY-364947; Sigma-Aldrich; the Netherlands) dissolved in serum-free medium. Cells were stimulated with 1 ng/ml of TGF-β_1 (kindly provided by Ken Iwata, OSI Pharmaceuticals, Melville, NY, USA) for 45 min. Cells were lysed with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, 20% (v/v) glycerol, and 8% (v/v) β-mercaptoethanol and stored at -20ºC for protein analysis.

Losartan treatment and AON transfection

Losartan potassium (Merck Sharp & Dohme BV) was dissolved in differentiation medium. KM109/DL589.2 cells were differentiated in 6-wells plates for 7-10 days before transfection. Losartan was added at concentrations ranging from 0 to 50 µg/mL and maintained during and after transfection. Cells were transfected with 500 pmol h49AON1 targeting exon 49 or h50AON1 targeting exon 50 (2'-*O*-methyl RNA oligonucleotides with a full-length phosphorothioate backbone (2OMePS) previously described[176]; Prosensa Therapeutics; the Netherlands) in 1 mL differentiation medium for 4 hours using 2 µL polyethylenimine (PEI, Exgen 500; MBI Fermentas; Germany) per µg AON, according to the manufacturer's instructions. Cells were harvested >24 hours after transfection.

In vivo *losartan and 23AON treatment*

All experiments were approved by and performed following the guidelines of the local animal ethics experimental committee of the Leiden University Medical Center (permit number: 08224). Mice were housed in individually ventilated cages in the animal facility of the LUMC and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD^{mdx}/J) with one or two copies of the utrophin gene (*mdx*/*Utrn*$^{+/-}$ or *mdx*/*Utrn*$^{+/+}$) were obtained from our own breeding facilities.

Four weeks old mice were treated orally (via the drinking water) with 0.6 g/L losartan potassium (Sigma-Aldrich) till the end of the experiments. Control mice received normal drinking water.

For intramuscular 23AON experiments, *mdx* mice (M/F) were, after 6 weeks of losartan/control treatment, anesthetized with isoflurane and intramuscularly injected in both gastrocnemius muscles on two consecutive days with 2.9 nmol (≡20 µg) of M23D(+2–18), a 2OMePS RNA oligonucleotide specifically targeting exon 23,[197] in 40 µL saline (*n*=2-3 per

condition). Mice were sacrificed 10 days after the last injection by cervical dislocation and muscles were isolated.

For systemic 23AON experiments, *mdx*/*Utrn*$^{+/-}$ mice (M/F) were treated for 8 weeks with subcutaneous injections of saline, losartan in drinking water, subcutaneous injections of 23AON or losartan in drinking water and subcutaneous injections of 23AON (n=3-4 per group). 200 mg/kg body weight/week M23D(+2–18) divided over 4 injections was injected subcutaneously. During treatment functional performance was tested weekly by rotarod and two limb hanging wire testing and in the last week by grip strength testing.[717] Blood samples were taken weekly via the tail vein for creatine kinase (CK) level measurements. Mice were sacrificed 10 days after the last 23AON injection and muscles were isolated, snap frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -80ºC.

Creatine kinase level measurements

Blood samples were centrifuged at 18 000 *g* for 5 min at 4ºC to generate plasma. CK levels were measured after diluting the samples 10 times in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen; CA, USA) and were measured with a Reflotron system (Roche Diagnostics; Switzerland) with CK-strips (Roche).

RNA isolation and exon skipping analysis by RT-PCR

For RNA isolation harvested cells were lysed with RNA-Bee (Campro Scientific; the Netherlands) and total RNA was extracted. RT-PCR analysis was performed using Transcriptor reverse transcriptase polymerase (Roche) in a 20 µL volume at 55ºC for 30 min with an appropriate primer. cDNA was amplified by nested PCR. Three microliters of cDNA was amplified in a 25 µL reaction for 20 cycles of 94ºC (40 sec), 60ºC (40 sec) and 72ºC (80 sec), followed by 32 cycles of 94ºC (40 sec), 60ºC (40 sec) and 72ºC (60 sec), with 1.5 µl of PCR product in a 50 µL reaction.

Muscles were minced in RNA-Bee (Campro Scientific) using MagNA lyser green beads (Roche) according to manufacturer's instructions. Total RNA was extracted and 1 µg of RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µl at 42ºC for 45 min with random hexamer primers (20 ng/µl). Then, 1.5 µl of cDNA was amplified in a 50 µL reaction for 30 cycles of 94ºC (30 sec), 60ºC (30 sec) and 72ºC (30 sec), as previously described.[663] All PCR products were visualized on 1.5% agarose gels and exon skipping levels were quantified using a DNA 1000 LabChip on the Agilent 2100 bioanalyzer (Agilent Technologies; CA, USA) according to the manufacturer's instructions.

Protein extraction and Western blot analysis

Cells were lysed with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% w/v sodium dodecyl sulphate (SDS), 0.1% w/v bromophenol blue, 20% v/v glycerol and 8% v/v β-mercaptoethanol. Muscles were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5). 50 mM NaCl, 1 mM EDTA, 1% Triton X100, 0.1% SDS, cOmplete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche) using MagNA lyser green beads (Roche) according to manufacturer's instructions. Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific; IL, USA) according to manufacturer's instructions, samples containing equal amount of protein were made and mixed 1:1 with sample buffer. Protein lysates were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose mem-

branes (Amersham; Belgium). Membranes were blocked in TBST-5% milk for 45 min at 4°C and incubated in primary antibody overnight at 4°C, followed by incubation with IgG-horseradish peroxidase-conjugated secondary antibody for 1 h. The detection was performed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). As primary antibodies rabbit polyclonal anti-phosphorylated Smad2 (1:1000; Ludwig Institute for Cancer Research; Sweden); rabbit polyclonal anti-phosphorylated ERK1/2 (1:1 000; Cell Signaling Technology, Inc.), mouse monoclonal anti-α-actin (Sigma-Aldrich) and rabbit monoclonal anti-ERK1/2 (1:1 000; Cell Signaling Technology Inc.) were used. As secondary antibodies goat-anti-rabbit and goat-anti-mouse IgG-HRP (1:2 500; SantaCruz Biotechnology; Germany) were used.

Biomarker analysis

For biomarker analysis total RNA was purified with a NucleoSpin RNA II kit according to the manufacturer's instructions, including a DNase digestion (Macherey-Nagel; Germany). One microgram of RNA was used for cDNA synthesis using random hexamer primers (20 ng/µl) and BioScript (GC biotech; the Netherlands) in 20 µl at 70ºC for 10 min and 42ºC for 1 h. Gene expression levels were determined for *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *AT1* (angiotensin II type 1) receptor, *Ctgf* (connective tissue growth factor), *Collagen1a1* (collagen type I α1), *PAI1* (plasminogen activator inhibitor 1)/*Serpine1*, *Myogenin* and *TGF*-β_1 (transforming growth factor β_1) by real-time qPCR using 2 µL of 10x diluted cDNA, 1 µL of forward primer (0.8 pmol/µL), 1 µL of reverse primer (0.8 pmol/µL) and 4 µL SensiMix SYBR (GC biotech) per sample and the Roche LightCycler 480 (Roche) with a program consisting of 45 cycles of 95ºC (10 sec), 60ºC (30 sec) and 72ºC (20 sec). Relative expression was calculated with Lin-RegPCR quantitative PCR data analysis software, version 11.3.[700] *Gapdh*, stably expressed across all conditions, was used to correct for differences in cDNA input.

Histology

Sections of 8 µm were cut from the gastrocnemius muscle with a Shandon cryotome (Thermo Fisher Scientific Co.; PA, USA) on Superfrost Plus slides (Thermo Fisher Scientific, Menzel-Gläser; Germany) with a minimum interval of 240 µm between the sections. Slides were fixed for 5 min in ice-cold acetone and stained with Harris haematoxylin and eosin (H&E) (Sigma–Aldrich) according to conventional histological procedures. Pictures were taken at 5x magnifications with a Leica DC500 camera and Leica IM50 software connected to a Leica DM LB light microscope (Leica Microsystems; The Netherlands). For blending and background correction Adobe Photoshop CS3 version 10.0.1 was used. Freely available ImageJ software with the haematoxylin/eosin (H&E) colour deconvolution plugin (Rasband, W.S., ImageJ, US National Institutes of Health; MD, USA) was used to determine the fibrotic/necrotic percentage of the entire cross section.[717]

Results and Discussion

Since losartan has been shown to act by antagonising TGF-β signalling as indicated by attenuating its downstream targets, including phosphorylation of Smad2,[260] the effect of losartan on TGF-β-induced pSmad2 was analysed *in vitro*. Differentiated mouse myocytes (C2C12 cells), were serum starved overnight and incubated for one hour with different concentrations

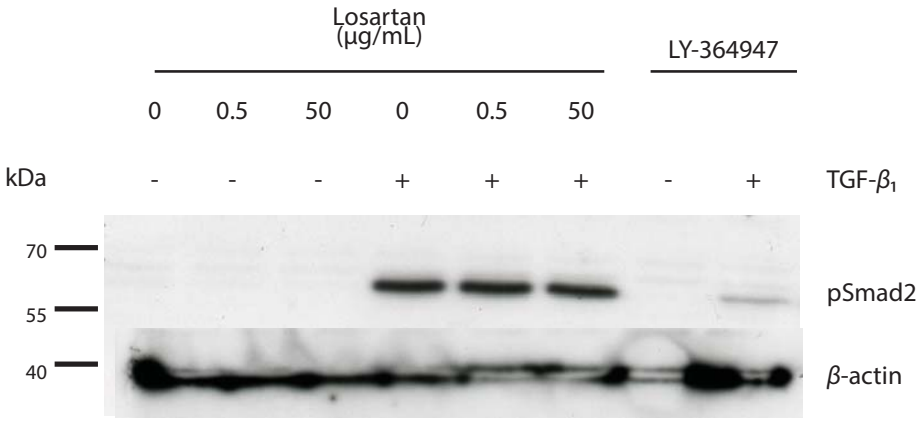

Fig. 7.1: Losartan does not influence Smad2 phosphorylation upon TGF-β_1 stimulation
A large increase in pSmad2 (60 kDa) is observed in stimulated muscle cells, which was not attenuated by pre-treatment with losartan. LY-364947, a TGF-β_1 receptor kinase inhibitor, was used as a positive control for TGF-β_1 inhibition. B-actin (42 kDa) was used as loading control.

of losartan before stimulation with TGF-β. pSmad2 analysis showed a potent induction of Smad2 phosphorylation upon TGF-β stimulation, which was not observed in non-TGF-β stimulated cells (fig. 7.1). Losartan did not attenuate this effect, which might be explained by the fact that no AT1 receptor expression could be detected in C2C12 cells (data not shown).

Healthy and DMD-derived muscle cells were treated with AONs targeting exon 49 and 50 in the absence and presence of varying concentrations of losartan. Losartan treatment did not influence exon skipping efficiencies (data not shown). Then the effect of losartan on local 23AON injection was studied. Four weeks old *mdx* mice were treated orally with losartan for six weeks after which they received two intramuscular injections with AONs against exon 23 in their gastrocnemius muscles. RNA analysis did not show differences in exon skipping levels between losartan-treated and control mice (fig. 7.2).

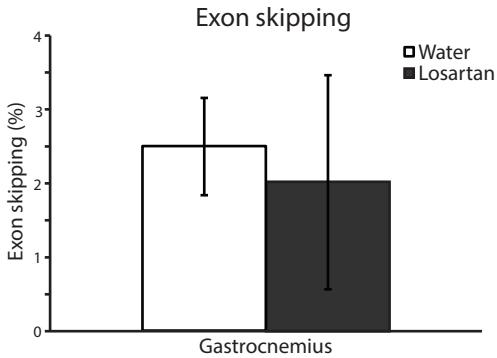

Fig. 7.2: Losartan has no effect on exon skipping levels after intramuscular injection of 23AONs in *mdx* mice
Quantification of exon 23 skipping levels after two intramuscular injections of 23AON in the gastrocnemius muscle. Six weeks pre-treatment with losartan did not change exon 23 skipping levels.
Data are represented as mean±SD.

To determine if there was any effect of losartan itself on signalling and muscle quality pSmad2 protein analysis and biomarker expression were performed after eight weeks of losartan treatment. pSmad2 protein might be reduced in losartan treated mice (fig. 7.3a), however group sizes were too small to draw conclusions. Expression pattern of most biomarkers did not differ between both groups in several skeletal muscles. Only a moderate reduction of some fibrotic markers (*i.e.* PAI1/Serpine1) was observed, while no change in TGF-β_1 expression itself was seen (fig. 7.3b-g).

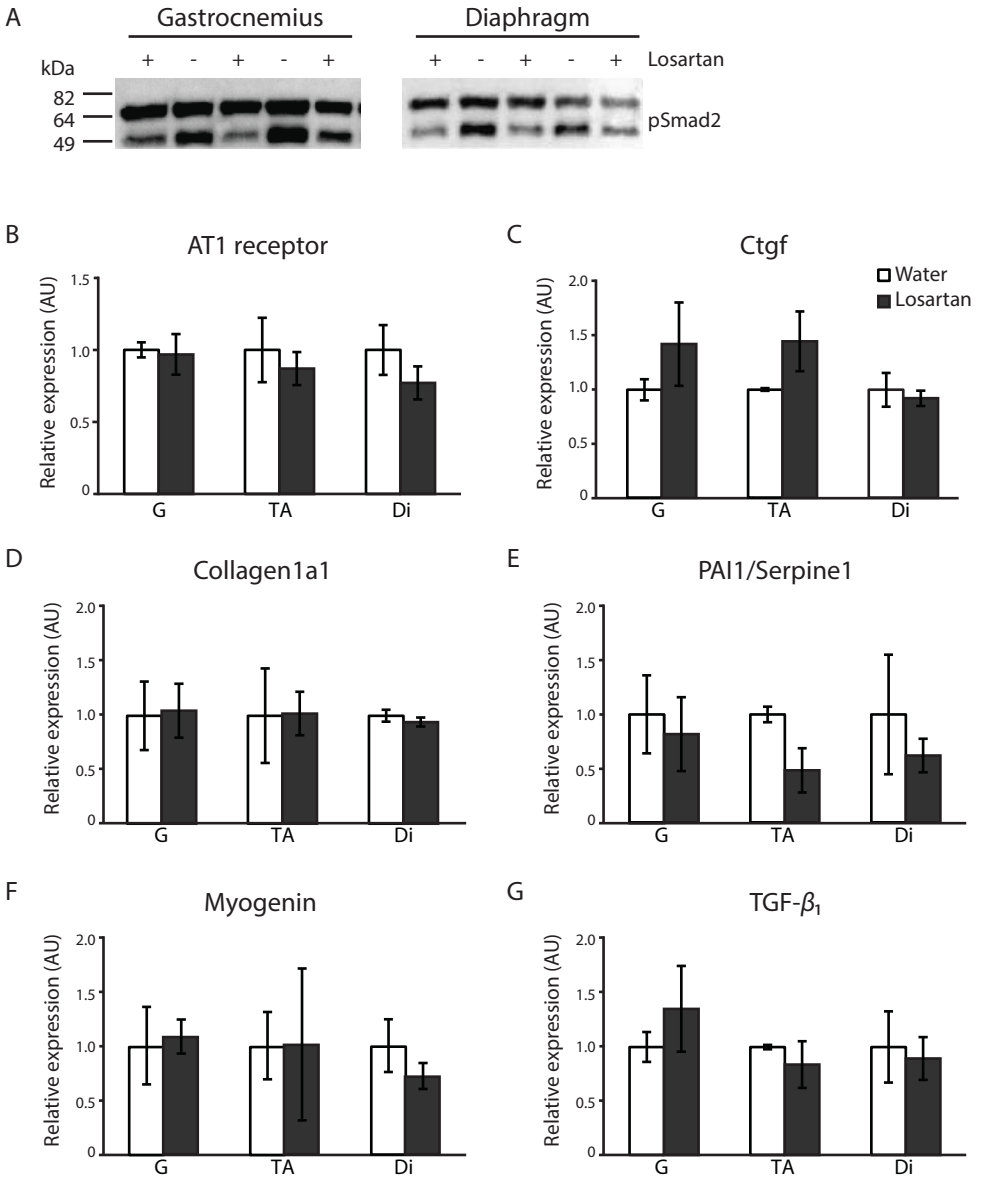

Fig. 7.3: pSmad2 protein levels and biomarker expression in losartan-treated *mdx* mice

a) After eight weeks of losartan treatment some reduction in pSmad2 protein expression (60 kDa; lower band) might be observed in the gastrocnemius and diaphragm of losartan-treated mice compared to control mice. However group sizes are small. b-g) Biomarker expression analysis showed no changes in several skeletal muscle in expression of the Angiotension type I receptor itself (b), several fibrotic markers (c-e), a regeneration marker (f) and TGF-β_1 (g), known to be differentially expressed in *mdx* mice. The housekeeping gene *Gapdh* was used to correct for differences in cDNA input and average expression is plotted relatively to control mice.

Error bars represent the SD. G=gastrocnemius; TA=tibialis anterior; Di=diaphragm; AT1=angiotensin II type 1 receptor; Ctgf=connective tissue growth factor; PAI1=plasminogen activator inhibitor 1; TGF=transforming growth factor

Thereafter the effect of losartan treatment on systemic 23AON treatment was analysed. Four weeks old *mdx*/*Utrn*$^{+/-}$ mice were treated for eight weeks with losartan, 23AON, a combination of both or control. *Mdx*/*Utrn*$^{+/-}$ mice were used, since pathology has shown to be aggravated compared to *mdx* mice, which display a relatively mild phenotype.[88] When we had included several animals in this experiment, additional reports on losartan in dystrophic mice were published that were much less promising than the initial publication. The first report showed only preservation of cardiac function after two years of treatment, but no effect on skeletal muscle.[261] Long term treatment did improve respiratory function, but increase of fore limb strength was only observed at two months of age and not at nine months.[263] Another group also did not observe improvement of skeletal muscle function, although a decrease in fibrosis was observed. They did see improvement of cardiac function, combined with decreased fibrosis in the heart.[264] Given these controversial results, we decided to perform an interim analysis to determine the effects of losartan itself. During treatment no changes in creatine kinase levels, a measure of muscle damage, and functional performance were observed between groups (data not shown). Histological analysis of the gastrocnemius by haematoxylin-eosin staining did not show attenuation of fibrosis by either losartan or 23AON treatment (fig. 7.4a/b).

Notably, the new literature also described that the positive effects observed in the first experiments were not mediated via blocking of TGF-β signalling via the AT1 receptor, but

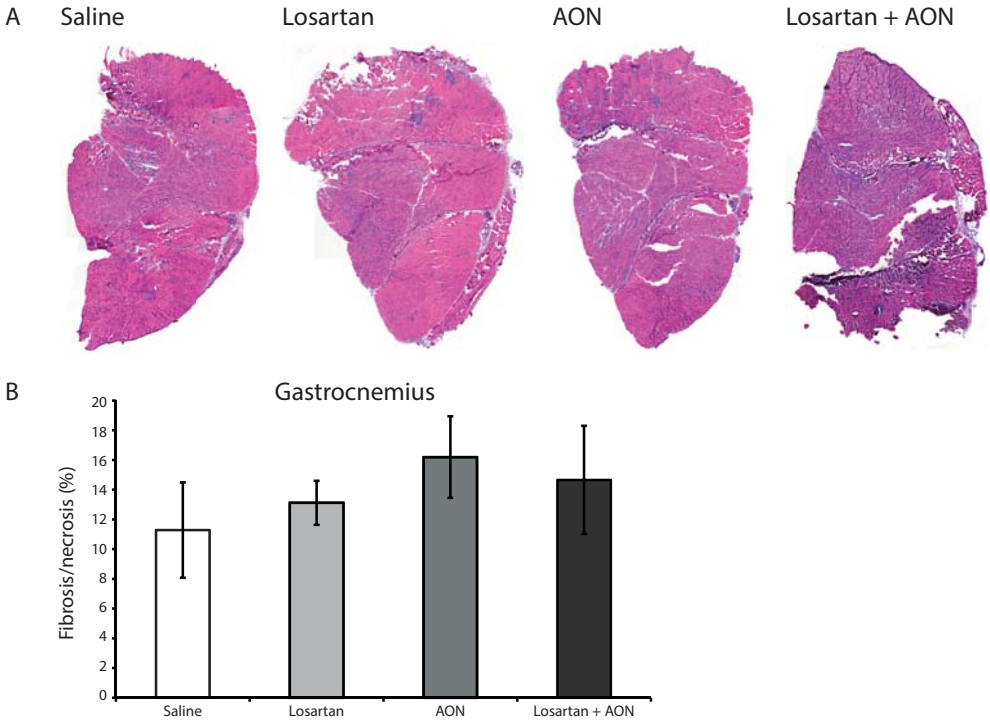

Fig. 7.4: Histological analysis of losartan and/or AON treated *mdx*/*Utrn*$^{+/-}$ mice.
No changes in fibrotic/necrotic area as measured by haematoxylin-eosin staining of the gastrocnemius muscle in mice treated with losartan and/or 23AON were observed.
a) Representative example for each group. Fibrotic/necrotic area is stained by haematoxylin (blue/purple), whereas eosin (pink) represents healthy tissue. b) Quantification of fibrotic/necrotic area.
Data are represented as mean±SD.

by stimulating the signalling via the AT2 receptor after AT1 receptor blockage, which has anti-fibrotic effects by blocking pERK.[262] Therefore, the effect of losartan on pERK1/2 was examined. Indeed in losartan treated cells a decrease in pERK1/2 (p44/p42 mitogen activated protein kinase or MAPK) was observed, independently of TGF-β stimulation (fig. 7.5a), however in mice results showed large variation between different mice, independently of treatment (fig. 7.5b). Furthermore in studies in animal models for other diseases, *e.g.* disuse atrophy, no changes were observed in either pSmad2 or pERK1/2 expression.[561]

In conclusion no effect of losartan on pSmad2 phosphorylation *in vitro* was observed. *In vivo* no signs of improved muscle quality, *e.g.* by histology or biomarker expression, were seen. These results and the building evidence in literature that the main, if any, beneficial effects of losartan are at cardiac function after long term treatment, made us decide to discontinue further treatment in combination with AONs. Hopefully in the future more effective muscle quality preserving agents will be discovered that can enhance the therapeutic effect of AON treatment.

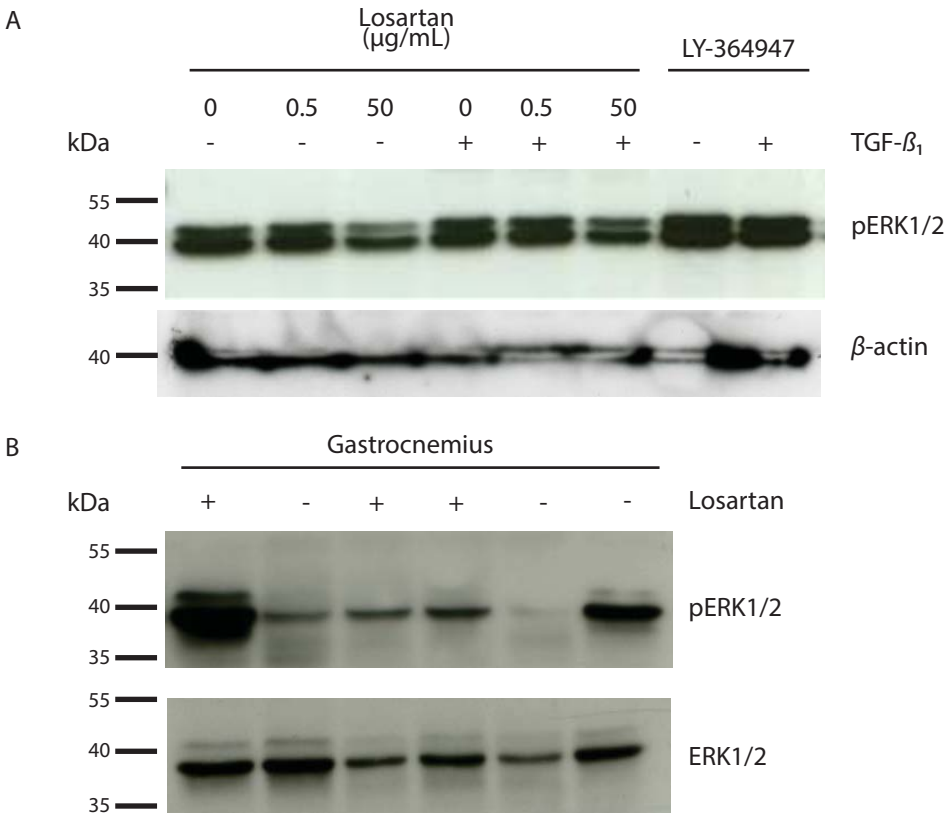

Fig. 7.5: Effect of losartan on ERK1/2 phosphorylation
a) In mouse myotubes a decrease in ERK1/2 phosphorylation (44/42 kDa) was observed, independently of TGF-β stimulation. This was not observed with a specific, a TGF-β_1 receptor kinase inhibitor (LY-364947). B-actin (42 kDa) was used as loading control. b) A large variation in pERK1/2 levels was observed between individual mice, showing no clear effect of losartan treatment versus control. Total ERK1/2 was used as a loading control.