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REGULATION OF ANGIOTENSIN II TYPE 1 RECEPTOR BY ITS MESSENGER RNA-BINDING PROTEINS

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ACADEMIC DISSERTATION

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II. Paukku K*, Backlund M*, De Boer RA, Kalkkinen N, Kontula KK, Lehtonen JY. Regulation of AT1R expression through HuR by insulin. *Nucleic Acids Research*. 2012;40(12):5250-5261.

III. Backlund M, Paukku K, Kontula KK, Lehtonen JY. Endoplasmic reticulum stress increases AT1R mRNA expression via TIA-1-dependent mechanism. *Nucleic Acids Research*. 2016;44(7):3095-3104.

* Equal contribution.

ABBREVIATIONS

AA	arachidonic acid
ACE	angiotensin converting enzyme
Ang	angiotensin
ARB	angiotensin II receptor blocker
ARE	adenylate-uridylate-rich element
ARE-BP	ARE-binding protein
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
ATF6	activating transcription factor 6
ATRAP	angiotensin II receptor-associated protein
AU	adenylate-uridylate
AUF1	AU-rich element RNA-binding protein 1, 37kDa
BP	blood pressure
cDNA	complementary DNA
Cox-2	cyclooxygenase 2
CRM1	chromosomal maintenance 1
CTGF	connective tissue growth factor
CVD	cardiovascular disease
DAG	diacylglycerol
eIF2α	eukaryotic translation initiation factor 2α
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ESC	European Society of Cardiology
ESH	European Society of Hypertension
FBS	fetal bovine serum
G3P	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRP78	glucose-regulated protein 78 kDa
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HuR	Hu antigen R
IL-6	interleukin 6
IP_3	inositol trisphosphate
IRE1α	inositol-requiring protein 1α
IRES	internal ribosomal entry site
IκB	inhibitor of NF-κB
JAK	Janus kinase
LDL	low-density lipoprotein
LMB	leptomysin B
MAPK	mitogen-activated protein kinase

MCP-1	monocyte chemoattractant protein-1
miRNA	microRNA
MK2	MAPK-activated protein kinase 2
MLCK	myosin light chain kinase
NAD	nicotinamide adenine dinucleotide
NOX	NADPH oxidase
NF-κB	nuclear factor κB
NO	nitric oxide
nt	nucleotide
O_2^-	superoxide anion
ONOO-	peroxynitrite
oxLDL	oxidized LDL
PCR	polymerase chain reaction
PERK	protein kinase RNA-like ER kinase
PI3K	phosphoinoside 3-kinase
PKC	protein kinase C
PKCδ	PKC isozyme δ
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
qPCR	quantitative polymerase chain reaction
RAS	renin-angiotensin system
REMSA	RNA electrophoretic mobility shift assay
RNBP	RNA-binding protein
ROS	reactive oxygen species
RRM	RNA recognition motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SG	stress granule
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
T2D	type 2 diabetes
TIA-1	TIA1 cytotoxic granule-associated RNA-binding protein
TNFα	tumor necrosis factor α
uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell

ABSTRACT

The renin-angiotensin system (RAS) is a key regulator of blood pressure and electrolyte homeostasis. Most hemodynamic responses to angiotensin (Ang) II are mediated via angiotensin II type 1 receptor (AT1R). Posttranscriptional regulation is an important mechanism in the regulation of AT1R expression. The primary target for posttranscriptional regulation of AT1R is the 3'-untranslated region (UTR) of its mRNA. AT1R 3'UTR has multiple adenylate-uridylate-rich elements that are recognized by various RNA-binding proteins (RNBP). These RNBPs regulate the expression of their target mRNAs by affecting mRNA stability, conformation, subcellular localization, and translation.

The aim of this study was to identify novel AT1R 3'UTR-binding RNBPs and understand their physiological role in AT1R function. AT1R 3'UTR-associated RNBPs were isolated from human vascular smooth muscle cell lysates by protein affinity purification with AT1R 3'UTR as a probe. Mass spectrometric identification of the AT1R 3'UTR-binding proteins led to identification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hu antigen R (HuR) and TIA1 cytotoxic granule-associated RNA-binding protein (TIA-1).

Oxidative stress induced by hydrogen peroxide (H_2O_2) increases AT1R expression. GAPDH was identified as a novel AT1R mRNA binding protein. GAPDH binds to a hairpin-structure rich in adenosine and uridine in the proximal 1-100 region of the AT1R 3'UTR and suppresses AT1R translation. Interestingly, H_2O_2 promoted GAPDH dissociation from AT1R 3'UTR. Thus, loss of GAPDH-mediated inhibition explains oxidative stress-induced increase in AT1R expression.

Insulin increases AT1R expression via posttranscriptional mechanisms. Insulin was shown to stabilize AT1R mRNA in a 3'UTR-dependent manner. HuR was found to bind with AT1R 3'UTR and stabilize AT1R mRNA. Insulin was shown to induce nucleocytoplasmic translocation of HuR to cytoplasm. This translocation of HuR increased HuR binding to AT1R 3'UTR, leading to increased AT1R expression by stabilization of AT1R mRNA.

AT1R avoids the endoplasmic reticulum (ER) stress-mediated translational suppression. TIA-1 was shown to bind to the 3'UTR of AT1R mRNA and inhibit AT1R expression. Using fluorescent microscopy, TIA-1 was shown to normally colocalize with AT1R mRNA

in the cytoplasm but to dissociate from it during ER stress. The dissociated TIA-1 was directed to translationally-silenced stress granules (SG), while AT1R mRNA remained excluded from them. Thus, AT1R mRNA avoids aggregation to SGs and TIA-1-mediated translational suppression during ER stress.

In conclusion, the 3'UTR of AT1R mRNA mediates the regulation of AT1R expression by oxidative stress, insulin stimulation, and ER stress. AT1R expression is modified by altered protein-mRNA interactions by changes in RNBP localization or expression levels.

INTRODUCTION

Angiotensin (Ang) II is a central component of the renin-angiotensin system (RAS) that regulates blood pressure, electrolyte and body fluid homeostasis, as well as cardiovascular structure. Most of the deleterious effects of Ang II, including elevated blood pressure, production of reactive oxygen species and activation of pro-inflammatory pathways, are mediated by angiotensin II type 1 receptor (AT1R) (Touyz and Schiffrin 2000, Dinh et al. 2001, Mehta and Griendling 2007, Higuchi et al. 2007). The type 2 receptor expression is much more restricted and usually has counteracting effects on AT1R (Horiuchi et al. 1999, Dinh et al. 2001). Cardiovascular diseases (CVD) combined constitute the leading cause of death and disability globally (www.who.int/en/). The important role of AT1R in development of CVDs is supported by the beneficial effects obtained by pharmacological inhibition of AT1R function (Oparil et al. 2001, PROGRESS Collaborative Group 2001, Gradman et al. 2005).

AT1R expression is regulated at multiple levels from transcription to translation. A number of physiological factors have been found to regulate AT1R expression via a posttranscriptional mechanism. Growth factors (Nickenig and Murphy 1994), Ang II (Nickenig and Murphy 1996), and estrogen (Wu et al. 2003) have been established as negative, whereas low-density lipoprotein (LDL) (Nickenig et al. 1997) and insulin (Nickenig et al. 1998) as positive regulators of AT1R expression in a posttranscriptional manner. Thyroid hormone mediates posttranscriptionally both positive and negative effects on AT1R expression, depending on cell type (Fukuyama et al. 2003, Diniz et al. 2012). RNA-binding proteins (RNBP) identified to posttranscriptionally regulate AT1R expression via the 3'-untranslated region (UTR) of AT1R mRNA include AU-rich element RNA-binding protein 1, 37kDa (AUF1) (Pende et al. 1999), calreticulin (Nickenig et al 2002, Mueller et al. 2008), and P100 (Paukku et al. 2008). Apart from Ang II-mediated posttranscriptional regulation of AT1R expression by AUF1 (Pende et al. 1999) and calreticulin (Nickenig et al. 2002, Mueller 2008), detailed molecular mechanisms of AT1R posttranscriptional regulation remain poorly understood.

Comprehensive understanding of regulation of AT1R expression may provide new possibilities in CVD treatment. This thesis explores the posttranscriptional regulation of

AT1R expression in more detail at the level of molecular mechanisms. The aim was to identify novel AT1R 3'UTR-associated RNBPs, describe their effect on AT1R mRNA dynamics and AT1R expression, and to define the physiological factors and mechanisms regulating their function.

REVIEW OF THE LITERATURE

1. Pathogenesis of hypertension and atherosclerosis

Cardiovascular diseases (CVD) such as hypertension, atherosclerosis, coronary heart disease, heart failure, cerebrovascular disease, and peripheral artery disease constitute the leading cause of death and disability globally. It has been estimated that in 2012 around 17.5 million people died from CVDs and according to a global status report by the World Health Organization, 9.4 million deaths could be attributed to high blood pressure in 2010 (www.who.int/en/). The present day global obesity epidemic further worsens these numbers.

1.1. Pathogenesis of hypertension

According to the European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines, the clinical classification of hypertension is a condition where blood pressure (BP) is continuously 140/90 mmHg or higher (http://www.escardio.org/). Hypertension can be divided into two categories, primary or secondary. The majority of cases, around 95 %, are classified as primary hypertension where there is no obvious underlying medical cause (Carretero and Oparil 2000). Secondary hypertension is caused by an underlying condition such as the presence of renovascular disease or a tumor-secreting blood-pressure-elevating hormones (Chiong et al. 2008).

Primary hypertension is a multifactorial condition where both genetic and environmental factors influence the development of hypertension. While no single genetic defect has been found to be the cause of primary hypertension, the genetic impact on BP varies greatly. In a study by Kupper et al. (2005) the heritability of BP was calculated to be 32-57% for systolic and 31-63% for diastolic, depending on the evaluation criteria. In addition to genetic predisposition, environmental factors play a significant role in the development of hypertension. The major environmental risk factors for hypertension include high alcohol consumption, diminished aerobic physical exercise, and unhealthy diet such as excess use of sodium, saturated fats, or high-fructose corn syrup.

The first steps in prevention or treatment of hypertension involve lifestyle changes and if this is not enough, pharmacological intervention may be applied. The main classes of anti-hypertensive drugs, according to the ESH and ESC guidelines, include beta-blockers, diuretics, calcium (Ca^{2+}) antagonists, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARB), renin inhibitors, and mineralocorticoid receptor antagonists (http://www.escardio.org/). Of these, ARBs directly inhibit AT1R activation whereas inhibitors of renin and ACE prevent Ang II production and thus indirectly inhibit AT1R functions.

1.2. Pathogenesis of atherosclerosis

Atherosclerosis is characterized by the formation of atheromatous plaques in the tunica intima of the arteries, leading to the thickening and stiffening of the arterial wall. The growing plaques may cause stenosis that reduces blood flow, leading to ischemia. Rupture of the plaques in turn may cause myocardial infarction or stroke via thrombosis i.e. the formation of a blood clot. A thrombus may block the artery, resulting in the restriction of blood flow at the site of the rupture or, if dislodged, in distal arteries. Extensive studies to unravel the pathophysiological mechanisms of atherosclerosis have revealed it as a multifactorial and complex disease. While a great deal has been learned from various animal models and cell culture experiments, the pathophysiological mechanisms in humans are still largely unknown. What is known is that the progression of atherosclerosis takes decades and involves a complex interplay between extra- and intracellular lipid metabolism, inflammatory processes, endothelial dysfunction, and immunological responses (Lusis 2000, Libby et al. 2011, Weber and Noels 2011)

As stated by the response to retention model, subendothelial accumulation of apolipoprotein B-containing lipoproteins, including low-density lipoprotein (LDL) and apolipoprotein B remnants, and resulting inflammation are considered as key initiators of atherogenesis, i.e. the formation of the plaques (Williams and Tabas 1995, Tabas et al. 2007). There are, in addition, other pathways connected to the initiation of atherogenesis. According to the response to injury model, atherogenesis is initiated by physical injury of the endothelial layer of the vascular wall, thus promoting the adhesion of leukocytes and production of pro-inflammatory factors at the site of injury (Ross et al. 1977). As the majority of developing

plaques show no evidence of physical injury of the endothelial layer, this triggering pathway was later modified suggesting that the endothelial dysfunction may act as an enhancer of atherogenesis by increasing the endothelial permeability to lipoproteins (Ross 1999, Gimbrone 1999). The second pathway connected to initiation of atherosclerosis, known as the oxidative modification hypothesis, underlines the importance of oxidatively modified lipoproteins such as oxidized LDL (oxLDL) in the progression of atherogenesis. This is supported by the findings that oxidative modification of LDL, taking place primarily in the tunica intima, promotes its uptake by macrophages via their scavenger receptors and is required for many of the pro-atherogenic functions of LDL (Steinberg et al. 1989, Diaz et al. 1997).

While the key triggering mechanisms of atherogenesis or their combinations are under extensive research and discussion, the subsequent consequences follow the same scheme. One of the first steps in atherogenesis is characterized by the adhesion of circulating leukocytes, mainly monocytes, to the endothelial cells lining the inner wall of the arteries **(Figure 1 A-B)** (Gerrity 1981). In response to atherogenic stimuli, vascular cells release chemoattractant cytokines (Moore and Tabas 2011, Drechsler et al. 2015) and the endothelial cells begin to express adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), on their surface (O'Brien et al. 1993, Cybulsky et al. 2001, Galkina and Ley 2007). Leukocytes that are guided to the site by chemotaxis attach to the adhesion molecules, roll on the endothelial surface and finally migrate across the endothelial monolayer to the underlying tunica intima. When resident in the intima, monocytes, a subset of leukocytes, differentiate into macrophages that engulf modified lipoproteins, including oxLDL or enzymatically modified LDL, transforming them into lipid-loaded foam cells that serve as an important source of lipid (mostly cholesterol ester) and cell debris accumulation in the developing plaque **(Figure 1B)** (Gerrity 1981, Bentzon et al. 2014).

Another important step in the initiation of atherogenesis is the infiltration of vascular smooth muscle cells (VSMC) from tunica media to tunica intima. When resident in the intima, the VSMCs proliferate and secrete collagen and elastin. These extracellular matrix components produce a structure known as a fibrous cap that covers and stabilizes the developing plaque **(Figure 1 C)** (Libby et al. 2011). Dying cells, such as VSMCs and foam cells, under the fibrous cap lead to accumulation of extracellular cell debris, lipids, and even cholesterol

crystals at the central part of the developing plaque, forming a structure known as the necrotic core (Libby et al. 2011, Bentzon et al. 2014).

Ultimately, disruption of the fibrous cap leads to plaque rupture **(Figure 1 D)**. One of the key features rendering the plaque susceptible to rupture is the thinning of the fibrous cap. This is thought to be due to decreased matrix formation, especially reduced collagen content, as a response to increased apoptosis of the plaque resident VSMCs and macrophage-induced proteolytic degradation of the matrix components (Bentzon et al. 2014). Fractured plaque exposes pro-coagulant material of the plaque core to coagulants in the blood, leading to the formation of the thrombus that is responsible for the ultimate pathological consequences of atherosclerosis.
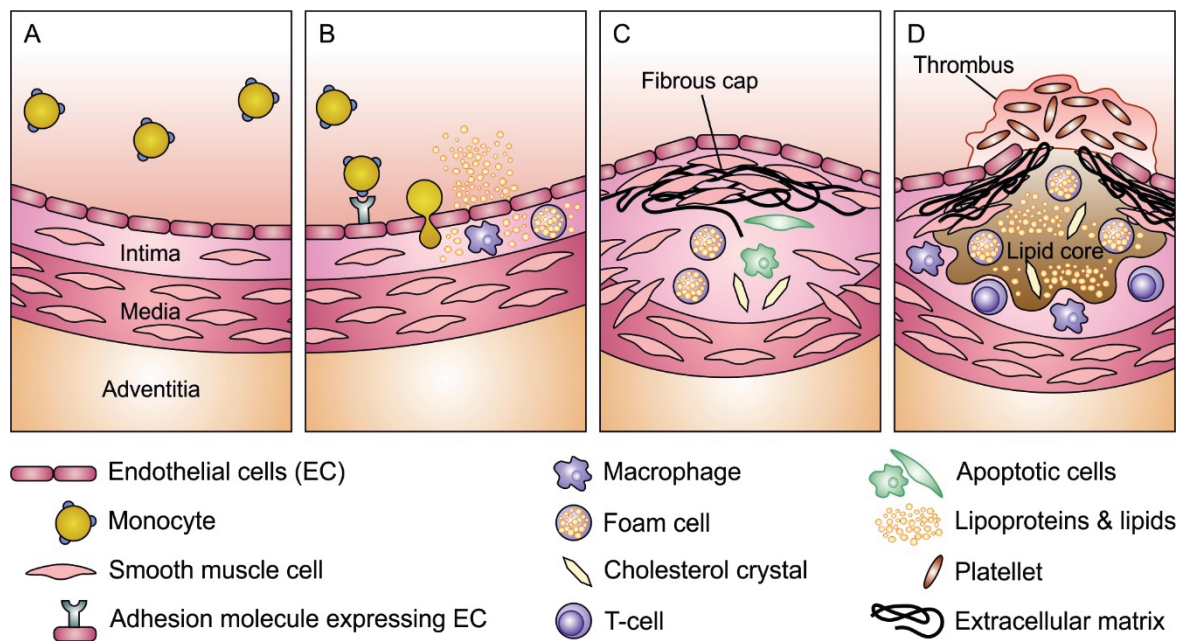
Figure 1. Atherogenesis. Adapted from Libby et al. 2011.

2. Regulation of gene expression at mRNA level

Cells regulate the expression of their genes in order to keep up normal physiological functions as well as to adapt to environmental changes. Gene expression is regulated at several different levels. Everything from the speed of transcription to the decay rate of the mRNA molecule are under strict control. The pre-mRNA molecules undergo extensive modifications such as the formation of the 5'-cap, addition of the poly-A tail and possible

splicing of introns and exons before being transported from the nucleus to the cytoplasm where they are taken up by the translational machinery (Proudfoot et al. 2002). In addition to these classical processes, there are several other regulatory steps between transcription and decay that are collectively referred to as posttranscriptional regulation.

2.1. mRNA structure and *cis*-acting elements

The three dimensional structure of an mRNA molecule is significantly more complex and versatile compared to a DNA molecule. The backbone of an RNA molecule consists of a phosphate group and a ribose sugar unlike the deoxyribose sugar in DNA molecules. Because RNA, unlike DNA, does not form double helix by pairing with a complementary RNA molecule, they can form different types of secondary structures within a single molecule. The secondary structures, such as hairpins and loops, are usually formed between complementary sequences within the mRNA by canonical Watson-Crick basepairing where cytosine and uracil pair up with guanine and adenine, respectively (Watson and Crick 1953). These structures play an important role in the regulation of RNA function. They may act as targets for various *trans*-acting factors such as RNA-binding proteins (RNBP) that have multiple roles in the posttranscriptional processes of mRNA molecules.

As a rule, mRNA structure can be divided into five different segments in 5'-3' order: 5'cap, 5'-untranslated region (UTR), protein coding sequence (CDS), 3'UTR and the poly-A tail **(Figure 2)**. The 5'-end of the transcript contains the 5'cap structure, the role of which is to guide the assembly of the translational machinery and protection of the mRNA transcript from exonuclease-mediated decay (Proudfoot et al. 2002). Following the cap, there is the 5'UTR that may regulate translational efficiency via various *cis*-acting elements such as internal ribosomal entry sites (IRES), which function in the cap-independent translation under situations like cellular stress when cap-dependent translation is halted (Kullmann et al. 2002, Holcik and Sonenberg 2005, Thakor and Holcik 2012). The presence of out-of-frame upstream open reading frames (uORF) allow the translation to initiate from an alternative site, thus competing with the translation from the actual start codon and resulting in reduced protein expression (Morris and Geballe 2000, Meijer and Thomas 2002, Calvo et al. 2009). When in-frame with the actual coding sequence, they may also produce isoforms varying in their N-terminal amino acid content (Martin et al. 2001)
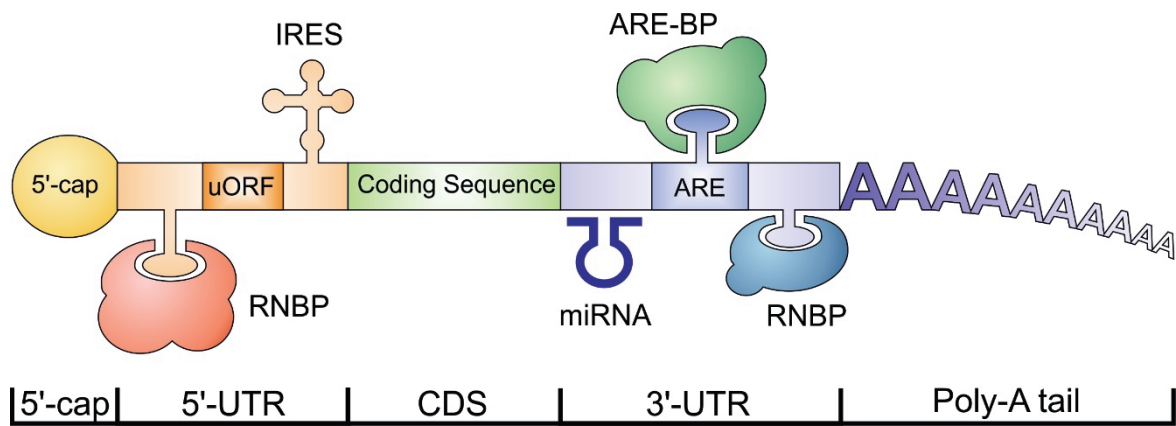
Figure 2. Structure of an mRNA molecule and its *trans*-acting factors. Adapted from Mignone et al. 2002.

The coding sequence located between the 5' and 3'UTR acts as a template for the encoded protein. The content of the coding sequence can be regulated via a process known as alternative splicing that can alter the actual polypeptide encoded by the mRNA. Following the coding sequence is the 3'UTR that is considered to be the major posttranscriptional regulatory element. While not being translated, this element functions as the target site for various regulatory mediators affecting the mRNA stability, conformation, nuclear export, translation, and localization. The *cis*-acting elements in the 3'UTR are specific sequences and structures that serve as targets for a variety of *trans*-acting factors such as RNBPs and microRNA (miRNA) molecules. Various *cis*-acting elements found in 3'UTRs are listed in **Table 1**.

Table 1. *Cis*-acting elements in 3'UTRs

CIS-acting element	Function	Example mRNA	Reference
AU-rich element (ARE)	Translation, stability	AT1R	Pende et al. 1999
GU-rich element (GRE)	Stability	c-jun	Vlasova et al. 2008
CU-rich element (CURE/DICE)	Translation, stability	MAP3K7IP2, r15-LOX	Wang et al. 2006, Messias et al. 2006
CA-rich element (CARE)	Splicing, stability	bcl-2, eNOS	Hui et al. 2003 Lee et al. 2009,
Iron responsive element (IRE)	Translation, stability	TfR	Casey et al. 1989
Selenocysteine insertion sequence (SECIS)	UGA to selenocysteine	GPx	Gromer et al. 2005

AT1R = angiotensin II type 1 receptor; AU = adenylate-uridylate; bcl-2 = B-cell CLL/lymphoma 2; CA = cytidylate-adenylate; c-jun = jun proto-oncogene; CU = cytidylate-uridylate; eNOS = endothelial nitric oxide synthase; GPx = Glutathione peroxidase; GU = guanylate-uridylate; MAP3K7IP2 = TGF-beta activated kinase 1/MAP3K7 binding protein 2; r15-LOX = reticulocyte 15-lipoxygenase; TfR = transferrin receptor.

The poly-A tail at the end of the transcript is added in a process known as polyadenylation, orchestrated by a multi-protein 3'-processing complex (Colgan and Manley 1997). The length of the poly-A tail can vary significantly but in mammals it is usually around 250 nucleotides (nt) in size (Kühn et al. 2009). In addition to protecting the transcript from exonuclease activity, the poly-A tail takes part in nuclear export of the molecule and in its translational regulation (Millevoi and Vagner 2010, Matoulkova et al. 2012). Genes may also contain more than one polyadenylation signal that may result in changes in the coding sequence or more commonly in the 3'UTR length of the transcripts by alternative polyadenylation (Tian et al. 2005, Elkon et al. 2013). This may have a significant effect on the 3'UTR-mediated regulatory mechanisms of the mRNA. For example, the genes encoding for Hu antigen R (HuR) (Al-Ahmadi et al. 2009) and cyclooxygenase 2 (Cox-2) (Hall-Pogar et al. 2005) contain alternative poly-A sites that result in variation of their 3UTR lengths and inclusion or exclusion of different 3'UTR-located adenylate-uridylate (AU) -rich elements (ARE).

2.2 AU-rich elements

The best characterized group of *cis*-acting elements in the 3'UTRs are the AREs. The current estimates for ARE-containing human genes vary between 5% and 16% (Bakheet et al. 2006, Gruber et al. 2011). AREs are usually described as destabilizing elements that promote decay of the mRNA molecule. However, depending on their associated *trans*-acting factors, they can lead to stabilization of the transcript as well (Lal et al. 2004, Barreau et al. 2006).

The core sequence of this element is AUUUA, but the classification is further divided into sub-categories depending on the more specified nucleotide sequence. The basic model from Chen and Shyu (Chen and Shyu 1994, Chen and Shyu 1995) divides AREs in three classes based on the variation of the core sequence. Class I AREs contain the classic AUUUA sequence in 1 to 3 copies within a uridylate-rich region, whereas the class II AREs consist of at least two overlapping UUAUUUA(U/A)(U/A) sequences. The class III AREs are not that well defined and can consist of uridylate rich regions lacking the AUUUA sequence. More detailed classifications, such as the one used by the ARE database, ARED (http://brp.kfshrc.edu.sa/ARED/) uses the classification of Chen and Shyu as the basis but divides the class II further depending on the number of the AUUUA repeats and their surrounding sequences (Bakheet et al. 2001, Barreau et al. 2006)

2.3 mRNA *trans*-acting factors

Multiple aspects of mRNA processing, such as nuclear export, posttranscriptional processing, subcellular localization, translation, and half-life are regulated by *trans*-acting factors like RNBPs that recognize their target mRNAs *cis*-acting elements via RNA-binding domains. The RNA-binding domains of different RNBPs consist of a variety of elements that are shared between the proteins, but each RNBP has its own combination of them. This facilitates the diverse functions and specificity of different RNBPs (Glisovic et al. 2008). One of the most abundant and widely studied domains is the RNA recognition motif (RRM), proposed to be present in up to 1% of human genes (Lunde et al. 2007). In addition, there are some unconventional protein domains that have been shown to possess RNA-binding ability but are yet to be classified as actual RNA-binding domains. An example of such a

structure is the NAD^+-binding domain of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that has been shown to bind AREs (Nagy and Rigby 1995, Nagy et al. 2000)

AREs are targets for a group of proteins called ARE-binding proteins (ARE-BP). They are a diverse group of proteins with multiple functions. They can either stabilize or destabilize the target mRNA as well as affect their translational efficiency. The effect of AREs on the mRNA depends on their associated ARE-BPs (Barreau et al. 2006). Some of the characterized ARE-BPs and their RNA-binding domains are listed in **table 2**.

ARE-BPs with opposite effects often compete for the same target mRNAs and one or the other is preferred, depending on environmental factors. For example, AUF1 and HuR both bind to the mRNA encoding for cyclin D1. HuR stabilizes the mRNA whilst AUF1 promotes its rapid degradation (Lal et al. 2004). HuR also competes with CELF2 (also known as CUGBP2) for binding to the 3'UTR of Cox-2 mRNA. Here, both HuR and CELF2 stabilize the mRNA, and while HuR enhances the translation, CELF2 has the opposite effect (Sureban et al. 2007). Cytochrome C expression is, in turn, regulated by HuR and TIA1 cytotoxic granule-associated RNA-binding protein (TIA-1) via 3'UTR-dependent translational control. While neither HuR nor TIA-1 affect cytochrome C mRNA stability, HuR promotes and TIA-1 inhibits its translation (Kawai et al. 2006).

In the coming sections only those ARE-BPs that are essential for the present thesis, i.e. GAPDH, HuR, and TIA-1, are described in more detail.

Table 2. Partial list of known ARE-BPs

ARE-BP	RNA-binding domains	Target example	Reference
AUF1	RNA recognition motif (RRM) x2	IL-6	Paschoud et al. 2006
CELF2	RRM x3	Cox-2	Mukhopadhyay et al. 2003
GAPDH	NAD$^+$-binding domain	Cox-2	Ikeda et al. 2012
hnRNP A1	RRM x2	cIAP1	Zhao et al. 2009
hnRNP A2	RRM x2	Glut 1	Hamilton et al. 1999
hnRNP L	RRM x2	Glut 1	Hamilton et al. 1999
Hsp70	ATP- and peptide-binding domains	Cox-2, VEGF	Kishor et al. 2013
HuR	RRM x3	Cox-2	Doller et al. 2008
KHSRP	K homology domain (KH) x4	LDLR	Li et al. 2009
Nucleolin	RRM x4	bcl-2	Sengupta et al. 2004
PTBP1	RRM x4	LDLR	Li et al. 2009
RNPC1	RRM x1	MIC-1	Yin et al. 2013
TIA-1	RRM x3	Cox-2	Dixon et al. 2003
TIAR	RRM x3	BRCA1	Podszywalow-Bartnicka et al. 2014
TTP	Zinc finger (Znf) CCCH-type x2	TNFα	Carballo et al. 1998
YBX1	Cold shock domain (CSD) x1	GM-CSF	Capowski et al. 2001

AUF1 = AU-rich element RNA-binding protein 1, 37kDa; bcl-2 = B-cell CLL/lymphoma 2; BRCA1 = breast cancer 1; CELF2 = CUGBP, Elav-like family member 2; cIAP1 = baculoviral IAP repeat containing 2; Cox-2 = cyclooxygenase 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Glut-1 = glucose transporter 1; GM-CSF = granulocyte-macrophage colony-stimulating factor; hnRNP A1 = heterogeneous nuclear ribonucleoprotein A1; hnRNP A2 = heterogeneous nuclear ribonucleoprotein A2/B1; hnRNP L = heterogeneous nuclear ribonucleoprotein L; Hsp70 = heat shock 70kDa protein 1A, HuR = Hu antigen R; IL-6 = interleukin 6, KHSRP = KH-type splicing regulatory protein; LDLR = low density lipoprotein receptor; MIC-1 = macrophage inhibitory cytokine-1; TIA-1 = TIA1 cytotoxic granule-associated RNA-binding protein; TIAR = TIA1 cytotoxic granule-associated RNA-binding protein-like 1; TNFα = tumor necrosis factor α; TTP = tristetraprolin; PTBP1 = polypyrimidine tract binding protein 1; VEGF = vascular endothelial growth factor; YBX1 = Y box binding protein 1; RNPC1 = RNA binding motif protein 38. The RNA-binding motifs are collected from the articles when possible or from the RNA-binding protein DataBase, RBDP (http://rbpdb.ccbr.utoronto.ca/).

2.3.1. GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is best known for its role in glycolysis where it catalyzes the conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3BPG) in a reaction that reduces nicotinamide adenine dinucleotide (NAD) from its oxidized form, NAD^+ to NADH. GAPDH is a multifunctional protein that takes part in several cellular processes (Nicholls et al. 2012). GAPDH localizes to the nucleus where it protects the cells from telomere shortening (Sundararaj et al. 2004, Demarse et al. 2009) and promotes apoptosis (Dastoor and Dreyer 2001, Hara et al. 2005, You et al. 2013). GAPDH also functions as a transcriptional regulator (Zheng et al. 2003, Harada et al. 2007) and interacts with other proteins, for example in regulation of protein transport from endoplasmic reticulum to Golgi (Tisdale 2001, Tisdale et al. 2004).

One of the best characterized extraglycolytic activities of GAPDH is its role as an RNBP. GAPDH binding to its target mRNAs is mediated by its NAD^+-binding domain and the GAPDH-RNA interaction may be competed and even inhibited by NAD^+, NADH, or ATP (Nagy and Rigby 1995, Nagy et al. 2000). Both connective tissue growth factor (CTGF) and Cox-2 mRNAs are targets of GAPDH; this interaction is inhibited by NAD^+ (Kondo et al. 2011, Ikeda et al. 2012). Kondo et al. also described the CTGF mRNA-GAPDH interaction to be decreased by an oxidative agent, diamide, thus suggesting a redox sensitive regulation of GAPDH binding to RNA (Kondo et al. 2011). This is further supported by redox-sensitive, 3'UTR-mediated regulation of endothelin-1 by GAPDH (Rodriguez-Pascual et al. 2008). Under normal conditions GAPDH acts as a negative regulator of endothelin-1 expression via increasing its mRNA decay. The active site of GAPDH is needed for RNA binding as both NAD^+ and G3P are capable of inhibiting the GAPDH-RNA interaction. Further, the catalytic cysteine residue at the active site of GAPDH regulates its redox-sensitive RNA-binding capability. Exposure of the wild type GAPDH to oxidative agents leads to modification of the active-site cysteine and inhibition of GAPDH-RNA interaction. Mutation of the GAPDH active-site cysteine to a serine fails to significantly affect the protein structure or the RNA binding capability but prevents the cysteine-targeted oxidative modifications. This mutated GAPDH retains its RNA-binding capability and suppressive function over enothelin-1 expression after exposure to the oxidative agents. This shows that

the active-site cysteine is regulating the redox-sensitive binding of GAPDH to RNAs (Rodriguez-Pascual et al. 2008).

2.3.2. HuR

Hu antigen R (HuR), also known as ELAV like RNA binding protein 1 (ELAVL1), belongs to a protein family of Hu-proteins consisting of three other family members, HuB, HuC, and HuD (Hinman and Lou 2008). HuR is a ubiquitously expressed protein that was first characterized and mapped by Ma et al. (Ma et al. 1996, Ma and Furneaux 1997). HuR consists of three RNA recognition motifs (RRM) RRM1, RRM2, and RRM3. RRM2 and RRM3 are separated by a hinge region that contains the HuR nucleocytoplasmic shuttling sequence (Fan and Steitz 1998a). RRM1 and RRM2 are thought to mediate RNA-binding, whereas RRM3 stabilizes protein-protein interactions and binds to the poly-A tail of mRNAs. HuR is primarily nuclear but upon various stimulations shuttles to the cytoplasm where it binds to its target mRNAs (Doller et al. 2008). HuR was originally described as an mRNA stabilizing factor that protects VEGF (Levy et al. 1998) and c-fos (Fan and Steitz 1998b, Peng et al. 1998) mRNAs from decay. Later, HuR was shown to stabilize a number of other ARE-containing mRNAs, including those for tumor necrosis factor α (TNFα) (Dean et al. 2001) and Cox-2 (Sengupta et al. 2003). In addition to promoting mRNA stability, HuR regulates alternative splicing (Izquierdo 2008, Zhao et al. 2014) and translation (Mazan-Mamczarz et al. 2003, Meng et al. 2005, Kawai et al. 2006). The detailed mechanisms by which HuR acts as a positive regulator of its target mRNAs is somewhat unclear but it has been suggested to involve regulation of polyadenylation (Dai et al. 2012), polysomal targeting (Doller et al. 2013), and competition with destabilizing factors (Linker et al. 2005, Sureban et al. 2007, Tiedje et al. 2012, Zhuang et al. 2013).

2.3.3. TIA-1

TIA1 cytotoxic granule-associated RNA-binding protein (TIA-1) was first characterized as a granule-associated protein in cytolytic lymphocytes and was originally designated as T-cell restricted intracellular antigen-1. It was identified to be expressed as two isoforms called p40-TIA-1 and p15-TIA-1 (Anderson et al. 1990, Tian et al. 1991, Kawakami et al. 1994). Later, the p15-TIA-1 isoform was actually shown to be transcribed by a distinct gene and

thus renamed as Granule Membrane Protein of 17 kDa (GMP-17), while the name TIA-1 was reserved for the p40 isoform (Medley et al. 1996). The gene encoding TIA-1 consists of 13 exons and there are two splice variants: TIA-1a that lacks exon 5 and TIA-1b where the exon 5 is included. Little is known about the functional differences between these two variants. They have been shown to exhibit differential tissue expression, and TIA-1b appears to have enhanced splicing activity over TIA-1a (Kawakami et al. 1994, Izquierdo and Valcárcel 2007).

In addition to its role in splicing, TIA-1 is associated with other cellular processes, including induction of apoptosis (Tian et al. 1991) and translational suppression (Dixon et al. 2003, Kawai et al. 2006). One of the best characterized functions of TIA-1 is its role in stress granule (SG) assembly in unfolded protein response (UPR) (Kedersha et al. 1999). TIA-1 is most abundantly found in the nucleus but constantly shuttles between the nucleus and cytoplasm (Zhang et al. 2005).

TIA-1 consists of three RRMs and a glutamine-rich C-terminal domain. The prion-related domain (PRD) in the C-terminus is needed for the aggregation of TIA-1 during SG assembly (Gilks et al. 2004). The three RRMs show distinct RNA-binding capabilities. The C-terminal RRM2 and RRM3 are needed for TIA-1 to bind uridine-rich sequences, where RRM3 is not required for the binding but stabilizes the interaction (Dember et al. 1996). The N-terminal RRM1 expresses no significant RNA-binding capability without the presence of the other RRMs. However, it has been shown to enhance the C-terminal domain-mediated protein-protein interaction between TIA-1 and U1-C protein of the U1 small nuclear ribonucleoprotein (Dember et al. 1996, Förch et al. 2002).

3. Cellular stress and the unfolded protein response

Cells are sensitive to different environmental changes and usually respond to them by adapting their metabolism accordingly. Nevertheless, sometimes the changes are too big or too long for the cells to cope with, leading to cellular stress. Oxidative stress, metabolic stress, and inflammation are common stressors that the cells are exposed to under various environmental conditions. The common denominator for all of them is the induction of endoplasmic reticulum (ER) stress and activation of UPR as an adaptive coping mechanism.

3.1. The unfolded protein response

Endoplasmic reticulum is the site of synthesis for secretory and membrane-bound proteins of the cell surface and intracellular compartments. In addition, it also participates in lipid metabolism, Ca^{2+} storage, and carbohydrate synthesis (Halperin et al. 2014). A situation where the homeostasis of the ER is compromised is known as ER stress. ER stress leads to the accumulation of unfolded proteins in the ER lumen and dissociation of an ER chaperone glucose-regulated protein 78 kDa (GRP78) from the luminal, N-terminal parts of three distinct ER transmembrane sensors: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α) and activating transcription factor 6 (ATF6) **(Figure 3)**. Dissociation of GRP78 from these sensors leads to their activation and to induction of UPR pathways to cope with and to overcome the unfolded protein load in the ER (Hetz 2012).
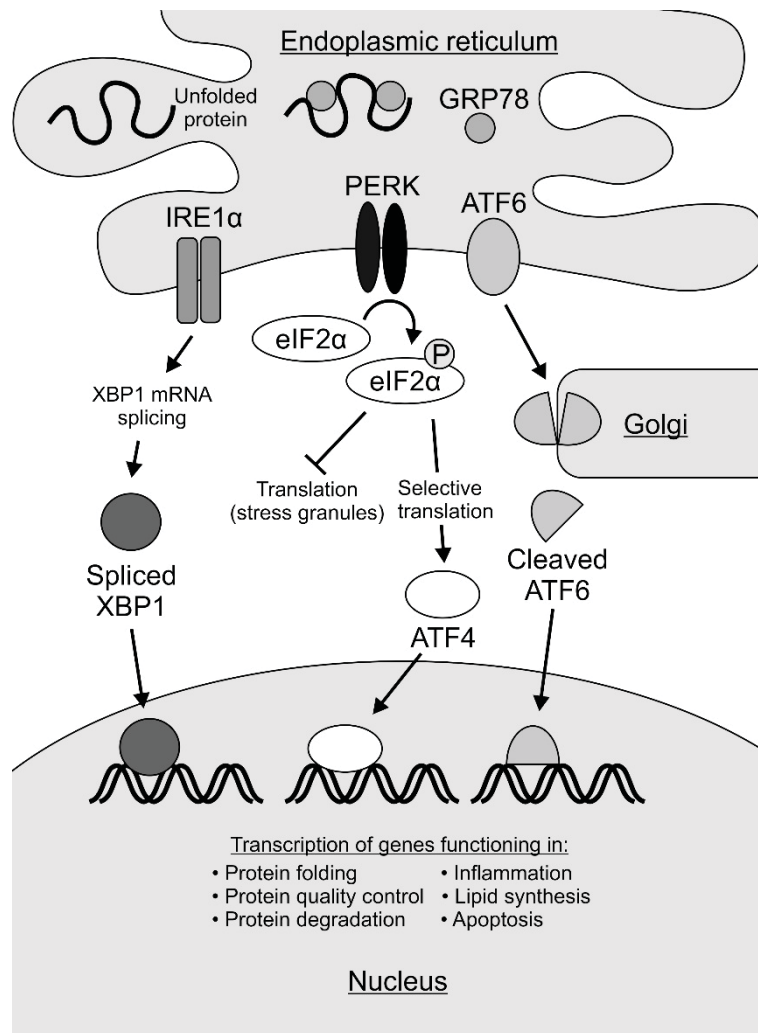

Figure 3. ER stress and UPR pathways. Adapted from Minamino et al. 2010.

Activation of IRE1α and ATF6 leads to activation of transcription factors upregulating the expression of various UPR genes. Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) leading to accumulation of non-functional translation initiation complexes. This inhibits the cap-dependent translation of new proteins, thus reducing the unfolded protein load in the ER. (Hetz 2012). Another consequence of eIF2α phosphorylation is the subsequent formation of SGs. These are macromolecular ribonucleoprotein-complexes consisting of polyadenylated mRNAs and proteins. The main function of these structures is to sequester mRNAs into translationally-silenced foci during cellular stress while protecting them from decay (Anderson and Kedersha 2009, Adjibade and Mazroui 2014).

3.2. Physiological stressors

Oxidative stress

Oxidative stress is a situation where the formation of reactive oxygen species (ROS) exceeds the capacity of the cells to neutralize them. ROS are needed for normal cellular signaling (Suzuki et al. 1997), but their excessive production has damaging effects on cells by oxidation of lipids, proteins, and nucleic acids, as well as by inactivating nitric oxide (NO). Some of the key sources for ROS within vascular cells are presented in **Figure 4**. NADPH oxidases (NOX) and xanthine oxidases, expressed in the cells at the vascular wall, are important sources of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Berry et al. 2000, Kelley et al. 2010). O_2^- reacts with the potent vasodilator NO, converting it to peroxynitrite ($ONOO^-$), a strong oxidant that has been suggested to promote atherogenesis *in vivo* by increasing LDL oxidation (Leeuwenburgh et al. 1997). During oxidative stress, uncoupling of the endothelial nitric oxide synthase (eNOS) may further reduce NO bioavailability. Uncoupling of eNOS switches its function from NO production to production of O_2^- (Münzel et al. 2005). This further increases the ROS load in the cell. While H_2O_2 alone is a relatively weak oxidant, it can promote the formation of hydroxyl radicals (OH•) by reacting with O_2^- or via a Fenton reaction where it oxidizes ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron (Maytin et al. 1999).
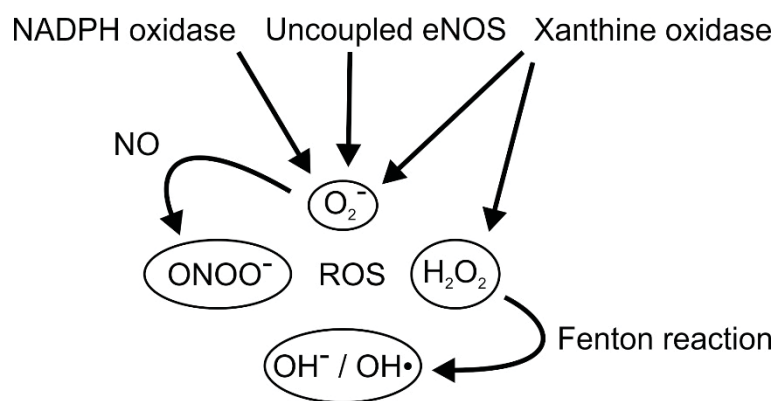
Figure 4. Production of ROS in the vasculature.

Normally antioxidants, including vitamin E and C or various enzymes, including superoxide dismutases, catalase, glutathione peroxidase, and peroxiredoxins are capable of circumventing the deleterious effects of ROS by neutralizing them. Superoxide dismutases catalyze the conversion of O_2^- to oxygen (O_2) and H_2O_2, which is further processed via catalase, glutathione peroxidase, and peroxiredoxins, to produce H_2O (Förstermann 2010). Under pathophysiological conditions when the ROS production is increased or prolonged, these enzymes are unable to cope with the ROS burden leading to pathological oxidative stress.

Metabolic stress

Metabolic stressors like hyperglycemia, hyperinsulinemia, and dyslipidemia may promote the formation of ROS and lead to ER stress and activation of UPR pathways.

Hyperglycemia is a situation where the blood glucose levels are too high. It is a common feature in type 2 diabetes (T2D) where the proper body insulin function is disrupted even though the insulin levels may be elevated. This is known as hyperinsulinemia or insulin resistance. Dyslipidemia refers to abnormal lipid levels in the blood and may be triggered by hyperinsulinemia. Relatively common examples of dyslipidemia are both hypercholesterolemia and hypertriglyceridemia combined with reduced high-density lipoprotein (HDL) levels.

Hyperinsulinemia has several mechanisms by which it can cause ER stress, and some of these are cell-type specific. It results in downregulation of insulin receptors on

macrophages, resulting in ER stress and apoptosis. The mechanism is mediated by inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) family and subsequent increase of cytosolic Ca^{2+} (Han et al. 2006, Liang et al. 2012). Compared to normal macrophages, insulin-resistant macrophages show increased ER stress and apoptosis following cholesterol loading. This is mediated via a transcription factor that increase the expression of a protein known as inhibitor of NF-κB (IκB). Nuclear factor κB (NF-κB) is a transcription factor and inhibition of NF-κB activity by IκB leads to decreased expression of various antiapoptotic and inflammatory genes under its control. This has been suggested to play a role in plaque development, necrotic core formation, and complications of atherosclerosis in diabetic individuals (Senokuchi et al. 2008).

Insulin resistance may reduce ER stress responses in adipose tissue via inhibition of the metabolic phosphoinoside 3-kinase (PI3K) pathway of insulin signaling. This pathway is inhibited in insulin resistant T2D patients or by lipid infusion in normal individuals (Boden et al. 2014). In the study by Boden et al. (2014), hyperinsulinemia, caused by hyperglycemia in normal individuals increased the expression of ER stress markers in the adipose tissue. However, no change in ER stress markers was seen if the PI3K-pathway was inhibited by lipid infusion. Similarly, in T2D patients with suppressed PI3K-pathway, no increase in ER stress markers was observed following insulin administration. Thus, it was proposed that insulin induces ER stress via PI3K-mediated metabolic pathways, affecting glucose metabolism and protein synthesis (Boden et al. 2014).

ER stress induced by obesity and inflammation has been suggested to promote hepatic insulin resistance that leads to increased gluconeogenesis and hyperglycemia, hallmarks of T2D (Kim et al. 2015). Insulin resistance in liver also causes an increase in lipogenesis that leads to elevated triglyceride-enriched very low-density lipoprotein (VLDL) secretion into circulation, which in turn promote development of atherosclerosis (Adiels et al. 2008).

In the vasculature and cells of vascular origin, hyperglycemia, hyperinsulinemia, and hyperlipidemia upregulate inflammatory biomarkers (Perkins et al. 2015). Hyperglycemia also induces nitric oxide synthase dysfunction, peroxynitrite production, and LDL oxidation (Tanaka et al. 2009). Further, in VSMCs, hyperglycemia induces ROS production via increased NADPH oxidase 4 (NOX4) expression (Xi et al. 2012). In VSMCs from a diabetic

rat model, hyperglycemia has in turn been shown to impair the regulation of intracellular Ca^{2+} sorting (Searls et al. 2010).

Inflammation

Inflammation and ER stress are closely linked. Several inflammatory factors may cause ER stress which further promotes the production of various pro-inflammatory molecules that attract inflammatory cells to the site of inflammation and thus amplify this positive feedback loop (Gargalovic et al. 2006, Zhang and Kaufman 2008).

NF-κB is a key regulator of the transcription of several inflammatory genes. It is activated during ER stress by phosphorylation of IκB that normally keeps NF-κB inactive (Pahl and Baeuerle 1995, Pahl and Baeuerle 1996, Jiang et al. 2003). Also, UPR-induced translational attenuation leads to increased NF-κB activation. The half-life of IκB is shorter than that of NF-κB and thus IκB is unable to suppress NF-κB in a situation where the translation of new proteins is halted (Deng et al. 2004). Dissociation of IκB from NF-κB leads to exposure of its nuclear-localization signal and nuclear accumulation where it regulates the transcription of its target genes (Beg et al. 1992).

Cytokines produced by inflammatory cells, such as TNFα, may stimulate ER stress. TNFα promotes intracellular ROS production thus leading to protein misfolding and ER stress (Xue et al. 2005). TNFα, together with interleukin 1β and interferon γ, has also been shown to induce NO accumulation and subsequent nitrosative stress via pathways involving NF-κB activation, signal transducer and activator of transcription (STAT) signaling, and nitric oxide synthase upregulation (Kacheva et al. 2011).

4. Renin-angiotensin system and angiotensin II type 1 receptor

4.1 Renin-angiotensin system

Blood pressure is regulated via a delicate signaling network comprising several organs and their hormonal products. Central in blood pressure regulation is the renin-angiotensin system (RAS) that is also involved in the regulation of fluid and electrolyte homeostasis.

The main organs involved in the RAS are kidneys, liver, and lungs. When blood volume is too low, the juxtaglomerular cells in the kidneys secrete renin that activates the conversion of liver-derived angiotensinogen to Ang I. Ang I is further processed to octapeptide Ang II by lung-derived ACE that removes two C-terminal peptides from the decapeptide precursor Ang I. Ang II is a vasoconstricting peptide that increases blood pressure by constricting the blood vessels. In addition, it stimulates the release of aldosterone from the adrenal cortex that acts by increasing sodium and fluid reabsorption in the kidneys, thus further elevating blood pressure via an increase of blood volume **(Figure 5)**.
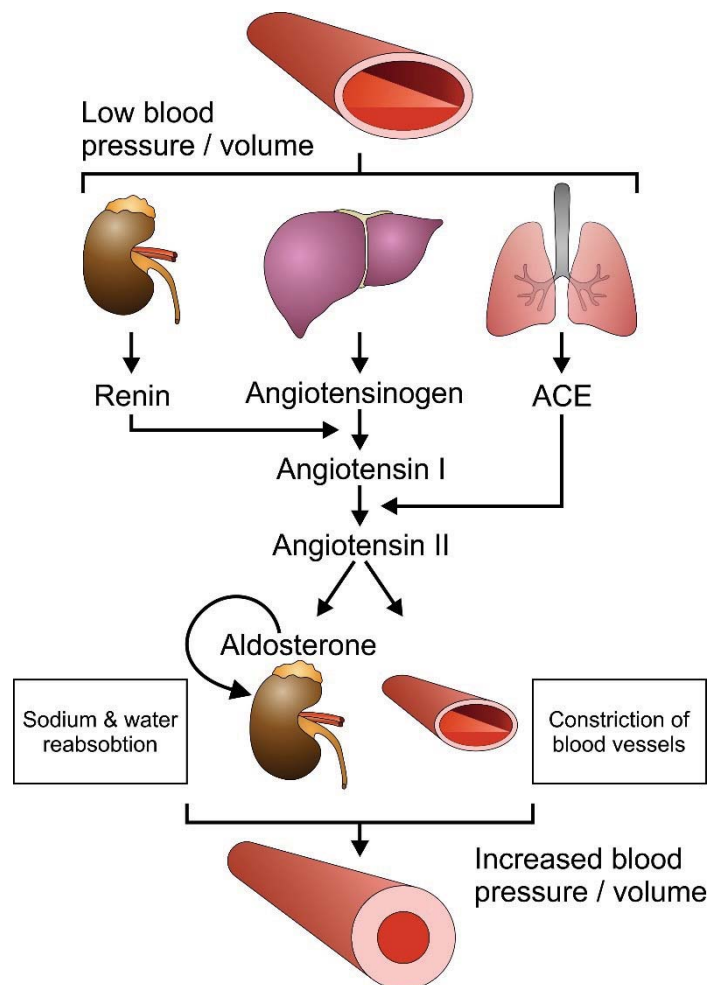

Figure 5. Renin-angiotensin system and blood pressure.

There are two different receptors for angiotensin II: AT1R and angiotensin II type 2 receptor (AT2R). Of these, AT1R is the predominant receptor that accounts for the deleterious effects of angiotensin II, whereas AT2R usually has counteracting effects (Stoll et al. 1995, Nakajima et al. 1995, Yamada et al. 1998, Horiuchi et al. 1999, Porrello et al. 2009).

4.2. AT1R signaling in vascular renin-angiotensin system

The Ang II-induced signaling via AT1R is mediated by activation of G-proteins coupled to the C-terminal tail of AT1R. These G-proteins can activate various signaling pathways that in the vasculature leads to vasoconstriction and transcription of various response genes **(Figure 6)**. AT1R signaling leads to activation of a number of effector molecules, such as phospholipases and protein kinases (Touyz and Schiffrin 2000, Dinh et al. 2001). The acute response to angiotensin II in VSMCs includes activation of the phospholipase C (PLC) - pathway that leads to formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate. IP_3 induces the release of Ca^{2+} from cellular compartments. DAG activates protein kinase C (PKC) that in turn activates the Na^+/H^+ exchanger affecting intracellular pH. Ang II further increases Na^+ influx by activating the Na^+-dependent Mg^{2+} exchanger. These events stimulate VSMC contraction by increasing myosin light chain phosphorylation and actin-myosin interaction. Myosin light chain is phosphorylated by myosin light chain kinase (MLCK). MLCK activation is primarily regulated by the IP_3-route of PLC-pathway. IP_3-mediated increase in intracellular Ca^{2+} leads to increased activation of calmodulin, a Ca^{2+}-dependent activator of MLCK. This, in turn, leads to increased VSMC contraction via increased phosphorylation of myosin light chain by MLCK (Touyz and Schiffrin 2000, Wynne et al. 2009, Lacolley et al. 2012).
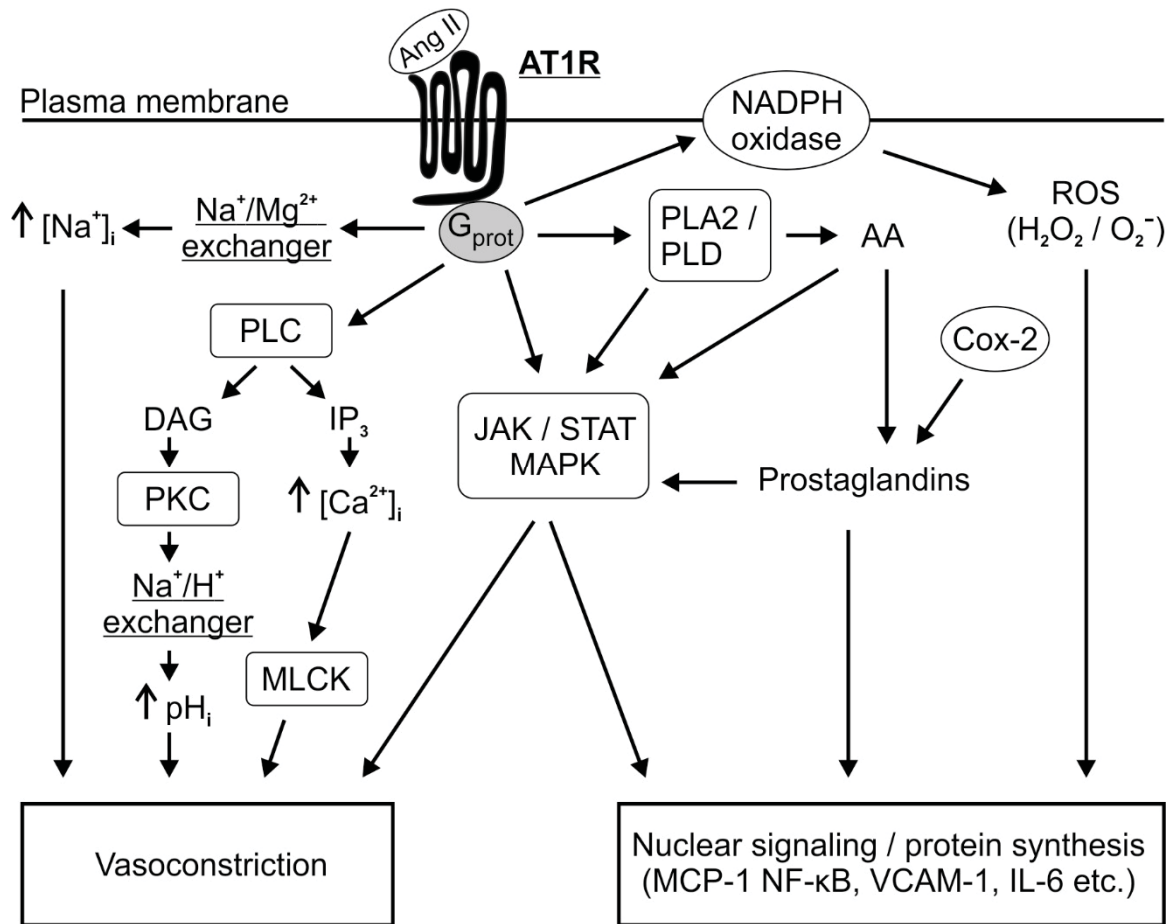
Figure 6. A schematic representation of some of the most important AT1R downstream signaling pathways in vascular cells. Adapted from Touyz and Schiffrin 2000.

The long term effects mediated by AT1R signaling include activation of various signal transducers, including Janus kinase (JAK), signal transducer and activator of transcription (STAT), and mitogen-activated protein kinases (MAPK) and their signaling pathways. Activation of phospholipases A2 (PLA2) and D (PLD) by AT1R increase the release of arachidonic acid (AA) which can be further metabolized to vasocoreactive prostaglandins, for example by Cox-2 (Touyz and Schiffrin 2000, Dinh et al. 2001, Hu et al. 2002). AT1R may also have a central role in cellular redox signaling and in the adverse effects of oxidative stress. Several studies show increased production of ROS via activation of vascular NOXs following Ang II administration (Griendling et al. 1994, Rajagopalan et al. 1996, Lassègue et al. 2001, Gragasin et al. 2003). The mechanism is suggested to be mediated via AT1R in vascular cells where the specific blockade of AT1R function inhibits Ang II-induced NOX activity (Rajagopalan et al. 1996, Gragasin et al. 2003).

Besides G-proteins the cytoplasmic tail of AT1R associates with several other proteins such as Cox-2 (Sood et al. 2014) and angiotensin II receptor-associated protein (ATRAP) (Daviet et al. 1999). ATRAP is a negative regulator of Ang II-induced AT1R signaling by promoting AT1R internalization (Daviet et al. 1999, Cui et al. 2000, Lopez-Ilasaca et al. 2003). In VSMCs Ang II induces Cox-2 expression and the mechanism involves AT1R internalization (Ohnaka et al. 2000, Hu et al. 2002, Morinelli et al. 2008). In HEK293 cells, elements in the cytoplasmic tail of AT1R have been shown to directly associate with Cox-2 and to downregulate its expression by ubiquitination and subsequent degradation (Sood et al. 2014).

In addition to the classical RAS components there are a number of variations adding a layer of complexity to the system. Several truncated peptides of Ang I or Ang II have been discovered. These include Ang 1-7, Ang 1-9, Ang 2-8 (also known as Ang III) and Ang 3-8 (also known as Ang IV). Ang 1-9 and Ang 1-7 are produced by angiotensin converting enzyme 2 (ACE2) -mediated removal of a single C-terminal peptide from Ang I or Ang II, respectively (Reudelhuber 2005, Fyhrquist and Saijonmaa 2008). The function of Ang 1-9 is poorly known. Some evidence suggests that it might act via AT2R to reduce blood pressure and have a beneficial effect on heart, blood vessels, and kidneys against cardiovascular remodeling due to hypertension or heart failure (Ocaranza et al. 2014). Ang 1-7 acts via binding to a receptor known as proto-oncogene mas (MAS1) and has been reported to act as a vasodilator and ACE inhibitor (Santos et al. 2003). Ang III is produced from Ang II by removal of an N-terminal peptide by aminopeptidase A and it can be further processed to produce Ang IV by aminopeptidase N. Ang III can bind to both AT1R and AT2R but with weaker affinity than Ang II (Reudelhuber 2005, Fyhrquist and Saijonmaa 2008). The receptor for Ang IV was first characterized in 1992 (Swanson et al. 1992), but it took nearly a decade before it was identified as the insulin-regulated aminopeptidase (IRAP) (Albiston et al. 2001).

In addition to the classical circulating RAS there is also a local tissue RAS that is found in several tissues and cell types. Among these, heart, vasculature, pancreas, brain, adipose tissue, and macrophages produce RAS components and are thus able to regulate RAS-mediated functions outside the control of the circulating RAS (Bader and Ganten 2008, Fleming et al. 2006).

5. Regulation of AT1R expression

AT1R was first cloned from rat (Murphy et al. 1991) and bovine (Sasaki et al. 1991), followed by cloning and characterization of the human AT1R complementary DNA (cDNA) (Furuta et al. 1992, Bergsma et al. 1992, Curnow et al. 1992). AT1R is a 41 kDa protein consisting of 359 amino acids. The gene encoding AT1R in humans is located in chromosome 3. It is a seven-transmembrane-spanning G-protein-coupled receptor with an extracellular N- and cytosolic C-terminus (de Gasparo et al. 2000).

5.1. AT1R transcript variants

The *AT1R* gene has been reported by NCBI to encode at least five different transcript variants (www.ncbi.nlm.nih.gov/gene/185). The variants consist of five alternative exons of which the last one contains the open reading frame for the functional protein whereas exons 1-4 are located in the 5'UTR. The five exons are of varying nucleotide length: exon 1 is 257 nt; exon 2 is 84 nt; exon 3 is 58 nt; exon 4 is 157 nt; and exon 5 is 2015 nt. The four noncoding 5' exons are present in various combinations together with the actual protein-coding exon 5 (2015 nt). The currently-reported exon organizations between the different transcript variants are 1/2/5 (variant 1), 1/5 (variant 2), 1/3/5 (variant 3), 1/2/3/5 (variant 4), and 3/4/5 (variant 5) (Warnecke et al. 1999, Elton and Martin 2007, http://www.ncbi.nlm.nih.gov/gene/185). The transcript variant 2 consisting of exons 1 and 5 encodes the predominant functional isoform. Exon 1 (257 nt) contains an IRES that functions in cap-independent translational activation during amino acid starvation-induced stress (Martin et al. 2003). Cellular stress is known to promote translation from uORFs and IRESs due to eIF2α phosphorylation (Vattem and Wek 2004, Holcik and Sonenberg 2005, Thakor and Holcik 2012).

Inclusion of exon 2 (84 nt) to the transcript in variants 1 and 4 creates two alternative uORFs that leads to decreased expression of AT1R via reduced translation (Warnecke et al. 1999). Before encountering a stop codon, the first uORF is predicted to encode a 7 amino-acid-long peptide and the second uORF, 11 and 9 amino acids in variants 1 and 4, respectively. It is thought that these uORFs are able to initiate translation, thus competing for ribosomes with the main initiation site in exon 5 (Martin et al. 2006).

Inclusion of exon 3 (58 nt) in the transcript creates two additional in-frame uORFs that could lead to incorporation of an extra 32 or 35 amino acids to the N-terminus of the protein. The nucleotides surrounding the most upstream start codon are not optimal considering translation initiation whereas the downstream uORF is in suboptimal context. This results in translation of AT1R isoform harboring 32 additional N-terminal amino acids (Curnow et al. 1995, Martin et al. 2001). As demonstrated by Martin et al., transcripts containing exon 3 are bicistronic, i.e. both the exon 3 and exon 5 start sites are used for translation and thus both the long and short receptor isoforms are produced from a single transcript (Martin et al. 2001). They further demonstrated that the binding of Ang II to the long isoform is weaker than to the predominant isoform and downstream signaling is activated at higher Ang II concentrations. This was hypothesized to allow fine-tuned regulation of Ang II responses by regulating the ratios of the two receptor isoforms. Moreover, as AT1R dimerizes with both AT1R and AT2R, creating homo and heterodimers, the long and short AT1R isoforms may add another level of regulatory complexity to the receptor function (AbdAlla et al. 2000, AbdAlla et al. 2001a, AbdAlla et al. 2001b).

The transcript variant harboring exon 3 and exon 4 (157 nt) is extremely rare and has been reported only from a human liver cDNA library (Guo et al. 1994). Taken together, these alternatively spliced isoforms enable a delegate fine-tuning, but at the same time increasing complexity, in the regulation of AT1R expression and function (Elton and Martin 2003, Elton and Martin 2007).

5.2. Posttranscriptional regulation of AT1R

AT1R expression is posttranscriptionally regulated by various physiological factors. Growth factors (Nickenig and Murphy 1994), Ang II (Nickenig and Murphy 1996), and estrogen (Wu et al. 2003) have been established as negative regulators of AT1R expression by a posttranscriptional mechanism. Thyroid hormones have been shown to decrease AT1R expression posttranscriptionally in VSMCs (Fukuyama et al. 2003) while increasing it in cardiomyocytes (Diniz et al. 2012). Other physiological factors that exert a positive impact on AT1R expression in posttranscriptional manner include LDL (Nickenig et al. 1997) and insulin (Nickenig et al. 1998).

The mRNA of AT1R carries an approximately 900 nt long 3'UTR that contains several AREs (Pende et al. 1999). A number of proteins and miRNAs have already been identified to regulate AT1R expression via these elements in a posttranscriptional manner (for AREs, see Section 2.2).

While the roles of these physiological factors, proteins, and miRNAs in AT1R regulation have been established, the exact detailed mechanisms of how they are interconnected remain poorly known. For the most part, the posttranscriptional regulators mediating the effects of the various physiological factors have not yet been identified. Similarly, the physiological factors regulating many of the identified *trans*-acting factors remain to be characterized (for *cis*-acting elements, see Section 2.1).

5.2.1. AUF1

AU-rich element RNA-binding protein 1, 37kDa (AUF1), also known as heterogeneous nuclear ribonucleoprotein D (HNRNPD), is best known for promoting the decay of ARE-containing mRNAs (DeMaria and Brewer 1996, White et al. 2013). AUF1 was the first RNBP to be identified as a 3'UTR-dependent posttranscriptional regulator of AT1R expression. AUF1 acts as a negative regulator of AT1R expression by decreasing the half-life of AT1R mRNA. Further, Ang II increases AUF1 expression, thus reinforcing its negative effect on AT1R (Pende et al. 1999).

5.2.2. Calreticulin

Calreticulin is another mediator of angiotensin II-induced destabilization of AT1R mRNA. Calreticulin is an ER-located protein classically described as a Ca^{2+}-binding protein regulating intracellular Ca^{2+} homeostasis and its ER storage capacity. In addition to ER localization, calreticulin can be found in other cellular compartments where it has multiple functions (Michalak et al. 2009, Gold et al. 2010). Ang II induces phosphorylation of calreticulin that enables its binding to the 3'UTR of AT1R. Similar to AUF1, calreticulin also negatively regulates AT1R expression by promoting the decay of its mRNA (Nickenig et al. 2002, Mueller et al. 2008). The experiments describing calreticulin in AT1R regulation

have been performed in vascular smooth muscle cells of rat origin and thus the mechanisms have not yet been validated in humans.

5.2.3. P100

P100, also known as staphylococcal nuclease and tudor domain containing 1 (SND1), is a component of the RNA-induced silencing complex (RISC). It functions in several aspects of mRNA processing including spliceosome assembly as well as the coactivation of several transcription factors (Yang et al. 2002, Paukku et al. 2003, Caudy et al. 2003, Scadden et al. 2005, Yang et al. 2007). P100 increases AT1R expression by 3'UTR-mediated stabilization and increased translation (Paukku et al. 2008). In addition to binding to AT1R 3'UTR under normal conditions, P100 also colocalizes with AT1R mRNA to SGs after heat shock or sodium arsenite treatment in HeLa cells (Gao et al. 2014).

5.2.4. MicroRNAs

In addition to RNBPs, certain miRNAs have been shown to target the 3'UTR of AT1R mRNA. TargetScan (http://www.targetscan.org/) predicts that AT1R mRNA may be a target for 302 miRNAs. The best characterized of the validated miRNAs is miR-155 that targets AT1R 3'UTR at a site containing a single nucleotide polymorphism (SNP) A1166C (rs5186). According to the Ensembl database (www.ensembl.org), the overall allele frequency for the minor allele is 0.118 but varies greatly between populations. The minor allele is most common among American (0.23) and European (0.27) populations whereas it is relatively rare in populations of African (0.02), East Asian (0.06), and South Asian (0.07) origin. The CC genotype of A1166C SNP is associated with increased aldosterone levels in hypertensive subjects and increased *in vitro* AT1R mRNA expression (Hannila-Handelberg et al. 2010). In large GWAS studies, however, no statistically significant association between *AT1R* SNPs and hypertension has been shown (Padmanabhan et al. 2015). The A allele-containing mRNA has been shown to be a target of miR-155-mediated downregulation, whereas the C allele leads to diminished interaction between the mRNA and miR-155 and thus increased AT1R expression (Zheng et al. 2010, Ceolotto et al. 2011, Haas et al. 2012). Further, miR-155 negatively affects Ang II-mediated VSMC viability and proliferation along with AT1R expression (Yang et al. 2014).

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AT1R is expressed in the human gastrointestinal tract where it is involved in regulation of fluid and electrolyte transport, as well as contractions of the colonic smooth muscle cells. AT1R expression is posttranscriptionally regulated in the human colorectal adenocarcinoma cell line, C2BBe1, by miR-802. This miRNA is expressed mostly in fetal colon and in adult colon as well, yet to a considerably lesser degree (Sansom et al. 2010).

Recently miR-410 has been shown to target the 3'UTR of AT1R mRNA in pancreatic cancer cells. The cancer tissue displays downregulation of miR-410 and upregulation of AT1R expression. The mechanism of miR-410 action is linked to suppression of cell growth, invasion, migration, and angiogenesis by suppressing AT1R expression (Guo et al. 2015).

6. AT1R pathophysiology

6.1. Vascular AT1R in hypertension

The vascular characteristics of chronic hypertension include vascular remodeling leading to stiffening and narrowing of the vessels due to increased media thickness (Intengan and Schiffrin 2001). Many hypertension-promoting cellular events are influenced by Ang II-mediated signaling pathways (Kim and Iwao 2000, Schiffrin and Touyz 2004, Mehta and Griendling 2007). This emphasizes the role of AT1R in the adverse effects of hypertension and is supported by the beneficial effects of anti-hypertensive drugs targeted to counteract AT1R function (Oparil et al. 2001, PROGRESS Collaborative Group 2001, Gradman et al. 2005).

The acute vasoconstricting response of AT1R may partly be explained by the PLC pathway, as discussed earlier (chapter 4.2.). Other mechanisms may take part in the prolonged regulation. One of the major intracellular consequences of AT1R signaling is the production of ROS, in particular that of O_2^-, which are central contributors to AT1R-mediated pro-hypertensive mechanisms. AT1R downstream signaling leads to activation of vascular NADPH oxidases, such as NOX1 on VSMCs or NOX4 on endothelial cells (Lassègue et al. 2001, Ago et al. 2004). The activation of the NOXs leads to production of O_2^-.

Accumulation of O_2^- in turn leads to inactivation of NO by conversion to $ONOO^-$, thus inhibiting the vasodilating effects of NO (de Gasparo 2002, Schulman et al. 2005).

In vitro studies using rat VSMCs suggest that NO inhibits the adverse effects of Ang II by suppressing AT1R expression (Ichiki et al. 1998). Another study with rat VSMCs further shows that Ang II increases VSMC migration and O_2^- production, and together with insulin reduces NO availability (Yang et al. 2005). Thus, the Ang II-induced scavenging of NO by ROS may further increase AT1R upregulation via attenuating the AT1R suppressive functions of NO. Taken together, these studies support the role of AT1R as a pro-hypertensive mediator. The central role of AT1R in regulation of blood pressure and development of hypertension is elegantly shown in animal models: mice expressing several copies of *AT1R*-gene or a constitutively active AT1R mutant develop hypertension, whereas AT1R knockout models show reduced blood pressure and response to Ang II (Ito et al. 1995, Le et al. 2003, Billet et al. 2007).

6.2. AT1R in atherosclerosis

There are several factors indicating a central role of AT1R in the progression of atherosclerosis, although the detailed mechanisms are poorly known. These include the role of AT1R in production of ROS, LDL oxidation and cellular uptake, production of pro-inflammatory factors, and expression of adhesion molecules on vessel wall. Ang II-mediated increase of ROS and decrease of NO leads to migration and infiltration of monocytes to the site of vascular endothelium under oxidative stress via increased expression of monocyte chemoattractant protein-1 (MCP-1) (Usui et al. 2000). Ang II-mediated signaling of AT1R further promotes the adhesion of monocytes to the vascular wall by increasing the expression of adhesion molecule VCAM-1 in endothelial cells (Pueyo et al. 2000).

Ang II and AT1R play a role also in the LDL-mediated adverse effects of atherogenesis. Normal and oxidized LDL upregulate AT1R expression in smooth muscle and endothelial cells of the vasculature, respectively (Nickenig et al. 1997, Li et al. 2000). Uptake of oxLDL by macrophages is further increased by AT1R-dependent upregulation of oxLDL receptor known as LOX-1 (Li et al. 1999, Morawietz et al. 1999). Accumulation of oxLDL in

macrophages leads to formation of cholesterol-ester-enriched foam cells that are present already in the early stages of atherosclerotic lesions (Libby et al. 2011). OxLDL also increases NF-κB expression and activation leading to increased transcription of pro-inflammatory genes, such as interleukin 6 (IL-6) and TNFα (Li et al.2005). Inflammation also attenuates HDL facilitated cholesterol removal from macrophage foam cells (Gillespie et al. 2015). Furthermore, increased AT1R expression due to oxLDL is linked to NF-κB activation (Li et al. 2000).

Hypertension is a significant risk factor for the development of atherosclerosis, and certain drugs used to treat hypertension have beneficial effects against atherogenesis in experimental setups. In an animal study, the AT1R antagonist losartan showed a beneficial effect on atherogenesis in atherosclerotic rabbits on a high fat diet. Losartan decreased the levels of inflammatory factors IL-6 and C-reactive protein as well as reduced thickening of the tunica intima (Xu et al. 2013). Importantly, serum lipid and Ang II concentrations were similarly increased in both losartan treated and untreated rabbits on a high fat diet. This indicates that the losartan-mediated anti-atherosclerotic effect is not mediated by reduction of lipid or Ang II levels, but may include reduction of NF-κB function, usually activated in response to Ang II. This limits the expression of NF-κB-regulated inflammatory cytokines and adhesion molecules. Further, in a study by Ishii et al. (2013), AT1R blockers olmesartan and valsartan reduced the volume of coronary atherosclerotic plaques in human patients by ca. 5% after six months of treatment. The reduction in plaque volume was accompanied by reduction in blood pressure. Whether the beneficial effects of the drugs were due to reduced blood pressure or more specific actions involving AT1R suppression remained unclear.

7. ER stress in atherosclerosis and hypertension

There is a growing amount of evidence showing that ER stress and UPR are associated with the development of atherosclerosis and hypertension (Tabas 2010, Minamino et al. 2010, Zhou and Tabas 2013, Santos et al. 2014). This is rational considering that many of the known risk factors and cellular responses associated with atherosclerosis and hypertension are also known ER stressors. Indeed, several ER stress markers are expressed in atherosclerotic lesions (Myoishi et al. 2007). Unstable plaques show increased expression

of ER stress markers as well as apoptotic smooth muscle cells and macrophages, which has been hypothesized to play a role in plaque vulnerability (Myoishi et al. 2007, Sanson et al. 2009). VSMCs apoptosis may promote plaque rupture as VSMCs produce collagen that normally stabilizes the fibrous cap (Geng and Libby 1995, Bauriedel et al. 1999). Another mechanism inducing plaque vulnerability involves the plaque resident inflammatory cells that secrete cytokines such as IL-6 that promote degradation of the extracellular matrix (Schieffer et al. 2000). Supporting this, in an atherosclerotic mouse model, VSMC apoptosis induced plaque vulnerability by reduced extracellular matrix, thinning of the fibrous cap, increased intimal inflammation, and accumulation of cell debris. VSMC apoptosis was linked to an increase of IL-6 levels and plaque MCP-1 expression (Clarke et al. 2006). In human VSMCs, OxLDL induces apoptosis by activating a pro-apoptotic signaling molecule, PKC isozyme δ (PKCδ). The mechanism is supposed to involve NOX-dependent production of ROS by oxLDL and activation of ER stress pathways. (Larroque-Cardoso et al. 2013). Though the detailed apoptosis-causing mechanisms in atherosclerosis are poorly understood, a chronic ER stress may be involved. Several ER stress-inducing agents have been described to induce VSMC and endothelial cell apoptosis *in vitro* (Scull and Tabas 2011). The results remain, however, to be validated *in vivo*.

ER stress also promotes inflammation associated with the progression of atherosclerosis. Free cholesterol accumulation in macrophages leads to ER stress and activation of pro-inflammatory NF-κB and MAPK pathways. This promotes inflammation by increased expression of inflammatory cytokines such as TNFα and IL-6 (Li et al. 2005). Oxidized phospholipids that accumulate in atherosclerotic lesions also trigger UPR and activate transcription factors needed for the expression of the various inflammatory cytokines (Gargalovic et al. 2006).

The direct association of ER stress with hypertension has mostly been shown by studies using animal models. In mice with Ang II-induced hypertension, inhibition of ER stress has beneficial effects. Chemical chaperones, used as ER stress inhibitors, reduce blood pressure, expression of ER stress markers, endothelial dysfunction and cardiac damage (Kassan et al. 2012). ER stress inhibitors also have beneficial effects on pulmonary artery hypertension in both rats and mice (Dromparis et al. 2013). Further, ER stress in the brain has been reported to promote Ang II-induced hypertension in mice. Increased ER chaperone expression in the

brain was shown to have a protective effect against both ER stress and hypertension (Young et al. 2012). The ER stress-induced hypertension by Ang II in the brain was later identified to be mediated via AT1R-dependent activation of transcription factor NF-κB (Young et al. 2015).

AIMS OF THE PRESENT STUDY

This study was aimed at identifying novel AT1R 3'UTR-associated RNA-binding proteins (RNBP) and to explore their mechanistic role as posttranscriptional effectors of AT1R mRNA and protein expression. The specific aims were as follows:

1. To identify novel AT1R 3'UTR-associated RNBPs involved in posttranscriptional regulation of AT1R expression (Studies I-III).

2. To investigate the posttranscriptional mechanisms by which insulin increases AT1R mRNA and protein expression (Study II).

3. To identify the physiological factors regulating the effects of the identified RNBPs on AT1R expression (Studies I and III).

4. To map the binding sites of the identified RNBPs on AT1R 3'UTR (Studies I-III).

5. To identify the posttranscriptional mechanisms, including regulation of mRNA stability, translation, and localization, by which the identified RNBPs mediate their action on AT1R expression (Studies I-III).

MATERIALS AND METHODS

Detailed descriptions of the materials and methods used in the different experiments can be found in the original publications I-III.

1. Cell culture studies

Cell culture

HEK293 cell line, derived from human embryonic kidney cells, was chosen as the principal experimental model since it is an immortalized cell line of human origin. HEK293 cells do not express AT1R, or express only minimally, which could interfere with the experiments via competition. When possible, data on HEK293 cells were validated in primary cells, human VSMCs. These cells do express AT1R which enables investigation of the mechanisms in an endogenous environment.

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), antibiotics, and glutamine. Early passage VSMCs were purchased from Lonza and grown in smooth muscle growth medium-2 with 5% FBS and supplements. To maintain the cell cultures, the sub-confluent cultures were detached from the culture plates with trypsin and split to new culture plates with appropriate seeding density. HEK293 cells were split twice a week with a 1:10 seeding density, whereas VSMCs were split approximately once a week with a 1:5 seeding density.

Transfections, transductions, and stimulations

Transfections were used to manipulate the expression of specific proteins in HEK293 cells. To overexpress the protein of interest, an expression plasmid was transfected to the cells using Fugene 6 or Fugene 6 HD transfection reagent. In order to silence the expression of specific proteins, siRNAs against its mRNA were transfected to the cells by Lipofectamine 2000. The cells were harvested 24-72 hours after the transfections for further analysis.

In studies II and III, expression levels of endogenous HuR or TIA-1 were altered by lentiviral vector systems. The use of lentiviral vectors enables the creation of stable cell lines with either over or downregulated expression of the protein of interest. In study II, both HuR-overexpressing and HuR-silenced VSMC cell lines were created using pLenti6 vector system (Sigma). In study III, TIA-1 expression was silenced in VSMCs by GIPZ lentiviral vector system (GE Healthcare).

Both HEK293 and VSMC cells were subjected to various chemical treatments, including H_2O_2, insulin, thapsigargin, and angiotensin II in the experiments.

2. Protein expression studies

Cell fractionation and protein extraction

The cells were lysed in appropriate lysing buffers or fractionated in cytoplasmic and nuclear extracts by a Cell Fractionation Kit (Pierce). The lysates were cleared by centrifugation and the protein concentrations were measured by Protein Assay kit (Bio Rad).

RNA probe preparation

In order to study binding and distribution of RNBPs on AT1R 3'UTR, either a full length or various fragments of the 3'UTR were synthesized *in vitro*. The RNA probes were prepared from cDNA templates by adding a T7 RNA polymerase promoter sequence to the 5'-end of the polymerase chain reaction (PCR) products. An additional 30-nt-long poly-A tail was added to the probes used in affinity purification. The PCR products were separated by agarose gel electrophoresis and extracted from the gel. The RNA probes were subsequently transcribed from the template by MEGAscript *in vitro* transcription system according to the manufacturer's instructions (Life Technologies). For probes to be used in RNA electrophoretic mobility shift assay, biotinylated UTP was used in the synthesis.

Affinity purification

Affinity purification was used to study protein-RNA interactions. In this method, the *in vitro* synthetizied AT1R 3'UTR RNA-probes, containing the poly-A tail, were coupled to poly-T linked Oligotex beads. The RNA probes were incubated in cell lysates after which the beads were added to the mixture. After extensive washing, the samples consisting of RNA probes connected to the beads and the proteins associated to the probes were subjected to either mass spectrometric identification or to western blotting.

Western blotting

To study protein expression, equal amounts of cell lysates or samples from affinity purifications were boiled in reducing Laemmli buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were subsequently transferred to a nitrocellulose membrane by semi-dry western blotting. After the blotting, the membrane was blocked for nonspecific binding and then subjected to the primary antibodies against the protein of interest. The unbound antibodies were washed off and the primary antibodies were detected by either biotinylated or fluorescently-labeled secondary antibodies. After final washing, the fluorescently labeled secondary antibodies were visualized using Odyssey scanner (Li-Cor). The biotinylated antibodies were subjected to streptavidin horseradish peroxidase-conjugate and the emitted light was exposed to an X-ray film.

Mass spectrometry

Proteins isolated by affinity purification were separated by SDS-PAGE and the gel was then subjected to silver (Study I and II) or Coomassie blue (Study III) staining to visualize the proteins. Protein bands of interest were cut out of the gel and digested in-gel to peptides with trypsin. The recovered peptides were subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF/TOF using Ultraflex TOF/TOF instrument (Bruker) for mass analysis. The protein identification from the acquired data was performed using Mascot Peptide Mass Fingerprint and Mascot MS/MS Ion Search programs (www.matrixscience.com).

Recombinant protein production and purification

In study III a recombinant MBP-TIA-1 fusion protein was produced to study the direct and GAPDH-dependent association of TIA-1 to the 3'UTR of AT1R mRNA. The fusion protein was produced in E.coli using TIA-1 cDNA and a pMAL Protein Fusion and Purification System (New England BioLabs).

Luciferase assay

Luciferase expression constructs were used in order to study the effect of AT1R 3'UTR on protein expression under various conditions in Studies I-III. The constructs consisted of a coding sequence for luciferase protein and various fragments or the full length 3'UTR of AT1R mRNA fused downstream of the coding sequence. When transfected to HEK293 cells the mRNA transcribed by the construct was subjected to the regulatory mechanisms mediated by the 3'UTR fragment present in the transcript. The produced luciferase protein could be quantified from cell lysates by photometric methods, where the luminescent signal produced by the luciferase protein was measured with a luminometer. To establish the role of the 3'UTR on protein expression, the results were normalized against measurements obtained from cells transfected with a luciferase construct lacking the 3'UTR.

Microscopy

Immunofluorescent microscopy was used to study localization and colocalization of endogenous proteins in VSMCs by confocal microscopy. The cells were grown on glass coverslips and fixed using paraformaldehyde. The proteins of interest were detected with specific antibodies that were further stained with fluorescently-labeled secondary antibodies. After the coverslips were mounted on microscopy slides the cells were visualized by a laser-scanning confocal microscope.

Immunofluorescent *in situ* hybridization was used to study the colocalization of endogenous AT1R mRNA and proteins. VSMCs were fixed on glass coverslips and hybridized with fluorescently-labeled RNA-probes against AT1R mRNA. After washing away the unbound probes, the samples were processed to facilitate immunofluorescent staining against the

protein of interest. The fluorescent labels were visualized with a conventional fluorescent microscope.

Ligand-binding assay

In studies I and III, a ligand-binding assay was used to determine AT1R expression on cell surface. Subconfluent VSMCs grown on 24-well culture plates were subjected to radiolabeled ^{125}I-[Sar^1,lle^8] Ang II (PerkinElmer) or unlabeled Ang II as control to quantify the membrane expressed AT1R. Following incubation with the normal or radiolabeled Ang II, the cells were washed with PBS containing 0.1% BSA and lysed in 0.5 N NaOH. The AT1R expression was quantified by measuring the radioactivity of the bound radiolabeled Ang II using a liquid scintillator. The results were normalized against the samples incubated with unlabeled Ang II.

In vitro **translation**

The direct role of GAPDH on AT1R mRNA translation was studied in Study I by *in vitro* translation assay. Here, a luciferase construct with or without AT1R 3'UTR was *in vitro* translated and biotinylated in the presence or absence of purified recombinant GAPDH. The translated protein product was assayed by luciferase method and by detection of the biotin label in western blots.

3. Protein-RNA interaction studies

Protein-RNA co-immunoprecipitation

Immunological methods were used to study protein-RNA interactions in Studies I-III. Cell lysates were prepeared using a lysis buffer that preserves the protein-RNA interactions (Peritz et al. 2006). The lysates were incubated with antibodies against the protein of interest. Protein G sepharose beads were added to the samples to bind the antibodies. After extensive washing, the proteins from the immunoprecipitated protein-RNA complexes were digested with proteinase K. The extracted RNA molecules were phenol-chloroform precipitated and subjected to expression profiling or reverse transcribed to cDNA.

RNA electrophoretic mobility shift assay

Changes in the protein-RNA interactions were explored in Studies I and II by RNA electrophoretic mobility shift assay (REMSA). Here, an *in vitro* synthesized and biotinylated RNA probe was added to cell lysates alone or together with an excess amount of unlabeled probe as competitor. Alternatively, an antibody against the protein of interest was added together with the biotinylated probe, in order to detect a specific protein-RNA complex by a band shift. Next the RNA, unbound to proteins, was digested with RNase, whereafter the samples were separated on a non-reducing SDS-PAGE and transferred to a nylon membrane. The biotin-labeled RNA was detected by chemiluminescence and observed for changes in the signal intensities or band shifts between different samples.

Random mutagenesis

In Study I, random mutagenesis was employed to detect the GAPDH-binding site at the 1-100 region of the AT1R 3'UTR. A library of luciferase reporter constructs containing the 1-100 3'UTR fragment with random mutations was created by GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies). From the created clones, those affecting protein expression were screened by luciferase assay. Binding of GAPDH to the mutated RNA was studied by affinity purification and REMSA.

4. mRNA expression studies

RNA isolation, cDNA synthesis and qPCR

In Studies I-III, quantitative polymerase chain reaction (qPCR) was used to quantify mRNA abundance or half-life in VSMCs or HEK293 cells. After appropriate treatments, the cells were harvested and the total RNA isolated using Nucleospin RNA II kit (Macherey-Nagel). The total RNA or RNA from protein-RNA immunoprecipitation was reverse transcribed to cDNA by Superscript II or III First Strand Synthesis System (Invitrogen) using oligo(dT)$_{12-18}$ primers. The expression of the gene of interest and appropriate controls were quantified by LightCycler (Roche) using Maxima SYBR Green (Fermentas) according to the manufacturer's instructions.

Expression profiling

In Study I, the precipitated RNA from protein-RNA co-immunoprecipitation was subjected to expression profiling in order to identify mRNAs coupled to immunoprecipitated GAPDH. The RNA was converted to biotinylated complementary RNA, hybridized to GeneChips and analyzed according to Affymetrix instructions.

RESULTS

1. Effect of the 3'UTR on AT1R expression

The regulatory effect of the AT1R 3'UTR on protein and mRNA expression was explored in Study I. HEK293 cells transfected with a luciferase construct containing the AT1R 3'UTR downstream showed a significant decrease in protein expression when compared to controls lacking the 3'UTR. This indicates that the 3'UTR is a negative regulator. This was accompanied by a comparable, 3'UTR-dependent decrease in luciferase mRNA expressions levels. Similar 3'UTR-dependent change in mRNA expression was observed when the luciferase coding sequence was replaced with AT1R coding sequence, thus confirming that the 3'UTR is indeed the regulatory element **(Figure 7)**. To further study the regulatory mechanisms by which the 3'UTR mediates its effect, both *in vitro* translation and luciferase mRNA half-life were measured in the presence and absence of the 3'UTR. Addition of the 3'UTR to the mRNA was observed to both increase the mRNA degradation and to suppress its translation **(Study I, Figures 1C and 1D)**.
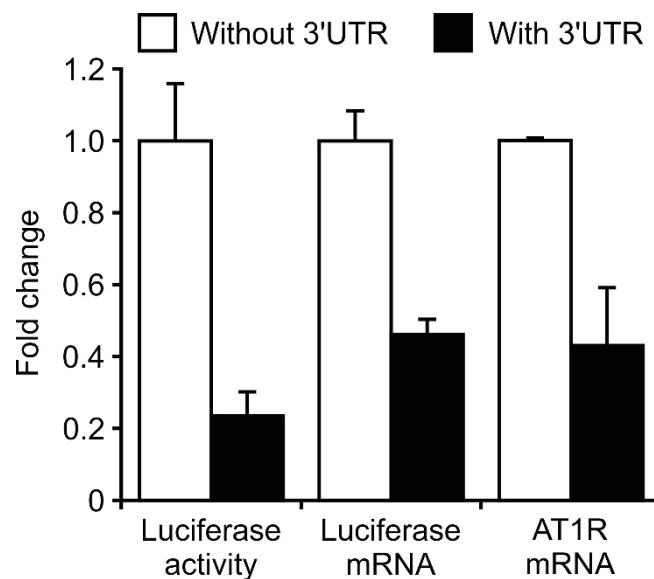
Figure 7. Effect of the AT1R 3'UTR on protein and mRNA expression. The results are shown as mean ± SD. A combination of data from Study I.

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2. Binding and distribution of RNBPs to AT1R 3'UTR

In studies I-III, three new RNBPs were identified to associate with the AT1R mRNA and regulate its expression via 3'UTR-mediated mechanisms. The identified proteins included GAPDH, HuR, and TIA-1. All of them are known RNBPs functioning in posttranscriptional regulation. The three proteins show distinct regulatory functions on AT1R expression.

The RNBPs were identified by mass spectrometry after affinity purification of RNBPs from cell lysates using probes consisting of the AT1R 3'UTR. In Study I, a protein at around 36 kDa range was observed to abundantly interact with various AT1R 3'UTR fragments and the protein was identified as GAPDH **(Study I, Figure 2B)**. In Study II, the insulin-mediated regulation of AT1R expression was explored. Another protein of around 36 kDa range was shown to exhibit increased association to the 3'UTR following insulin stimulation **(Study II, Figure 2A)**. This protein was subsequently identified as HuR. In Study III, a protein of around 40 kDa was shown to specifically associate with full length AT1R 3'UTR and was identified as TIA-1 **(Study III, Figure 1A)**.

Using affinity purification with various AT1R 3'UTR fragments and western blotting, the binding of the proteins to the 3'UTR was confirmed and the binding sites mapped **(Figure 8)**. In Study I, GAPDH was shown to bind the proximal region, spanning the 100 first nucleotides of the 3'UTR. In Study II, HuR was identified to bind a region spanning nucleotides 300-887 and in Study III, TIA-1 binding sites were mapped to two distinct locations, the proximal 1-100 and distal 600-887 fragments of the 3'UTR **(Figure 8)**.
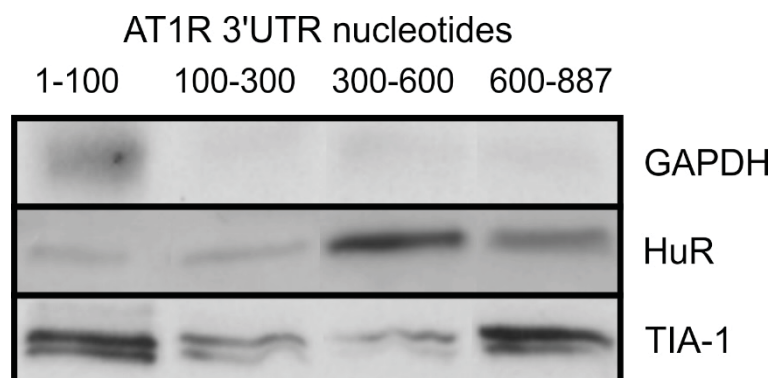

Figure 8. Binding and distribution of RNBPs to AT1R 3'UTR. Western blot images of GAPDH, HuR, and TIA-1 are displayed indicating binding to specific nucleotide regions. A combination of data from Studies I-III.

As observed by REMSA, the binding of GAPDH to the AT1R 3'UTR 1-100 region was direct as purified recombinant GAPDH did not require other proteins for the association **(Figure 9A)**. Deletion of nucleotides 9 and 11 from the 3'UTR were able to abolish GAPDH binding, identifying this as the GAPDH responsive element. Computational modelling of the AT1R 3'UTR 1-100 region suggested a hairpin-like structure that is disrupted by the 9/11 deletion **(Figure 9B)**. When analyzing the sequences from several GAPDH target mRNAs, the GAPDH motif was identified as an AU-rich sequence element **(Figure 9C)**.

Figure 9. GAPDH-binding motif of AT1R 3'UTR. (A) REMSA showing the binding of purified GAPDH to AT1R 3'UTR 1-100 region. (B) REMSA showing the inhibition of GAPDH binding to AT1R 3'UTR 1-100 by 9/11 deletion. The predicted structure of the wild type and deletion RNAs is shown. The deleted nucleotides are shown in red (1-100). (C) The consensus GAPDH target sequence. A combination of data from Study I.

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Insulin-induced association of HuR with AT1R mRNA was confirmed by immunoprecipitation coupled to qPCR from VSMCs (Study II). HuR was immunoprecipitated with a HuR-specific antibody from VSMCs before or after insulin stimulation and the associated AT1R mRNA was quantified by qPCR. At baseline, AT1R mRNA showed increased association to HuR when compared to a control where nonspecific IgG was used for immunoprecipitation. After insulin stimulation, AT1R mRNA association to HuR further increased when compared to the IgG control. No significant change in AT1R mRNA association was observed to other known AT1R 3'UTR-binding proteins in response to insulin stimulation **(Study II, Figure 2B)**.

Since GAPDH was identified to associate to the proximal 3'UTR region (Study I), the possible interaction between TIA-1 and GAPDH at this site was explored in Study III. TIA-1 binding to AT1R 3'UTR was shown to be GAPDH-dependent, as GAPDH silencing attenuated TIA-1 binding to the 1-100 region as observed by affinity purification, whereas TIA-1 silencing had no effect on GAPDH binding **(Study III, Figure 3A)**. Further, using purified GAPDH and TIA-1-MBP fusion protein in affinity purification, TIA-1 binding to the 1-100 region was increased by addition of GAPDH to the samples. GAPDH binding however, was not significantly dependent on the presence of TIA-1 **(Study III, Figure 3B)**. These data suggest that GAPDH binds to the AT1R 3'UTR directly, whereas TIA-1 requires GAPDH for the association at the proximal site. Using purified proteins in the affinity purification also demonstrated that TIA-1 and GAPDH do not require any other proteins for the association with the 3'UTR. TIA-1 binding to the distal 600-887 region was direct as GAPDH did not bind to this region in affinity purification from cell lysates nor was TIA-1 binding affected by GAPDH in affinity purification using purified proteins **(Study III, Figures 3A and 3B)**. To study endogenous protein-RNA interactions, fluorescent *in situ* hybridization against AT1R mRNA combined with immunofluorescent staining was used. In this experimental setup both TIA-1 and GAPDH were shown to colocalize with AT1R mRNA in VSMCs under normal conditions **(Study III, Figures 1D, 4C and 6A)**.

3. RNBP effects on AT1R expression

The mechanism by which GAPDH regulates AT1R expression was investigated in Study I, using a luciferase reporter gene expression system. GAPDH was identified as a negative regulator of AT1R expression via a 3'UTR-mediated mechanism. Following GAPDH silencing, a luciferase reporter gene exhibited an AT1R 3'UTR-dependent increase in luciferase protein expression in HEK293 cells, whereas the luciferase mRNA was reduced. Similar responses were seen in expression of both endogenous AT1R protein and mRNA in VSMCs **(Figure 10)**. This suggests that GAPDH stabilizes AT1R mRNA while suppressing its translation. As nucleotides 9 and 11 of the 3'UTR are needed for GAPDH binding, a luciferase construct lacking these nucleotides showed no significant response to GAPDH silencing **(Study I, Figure 4A)**.
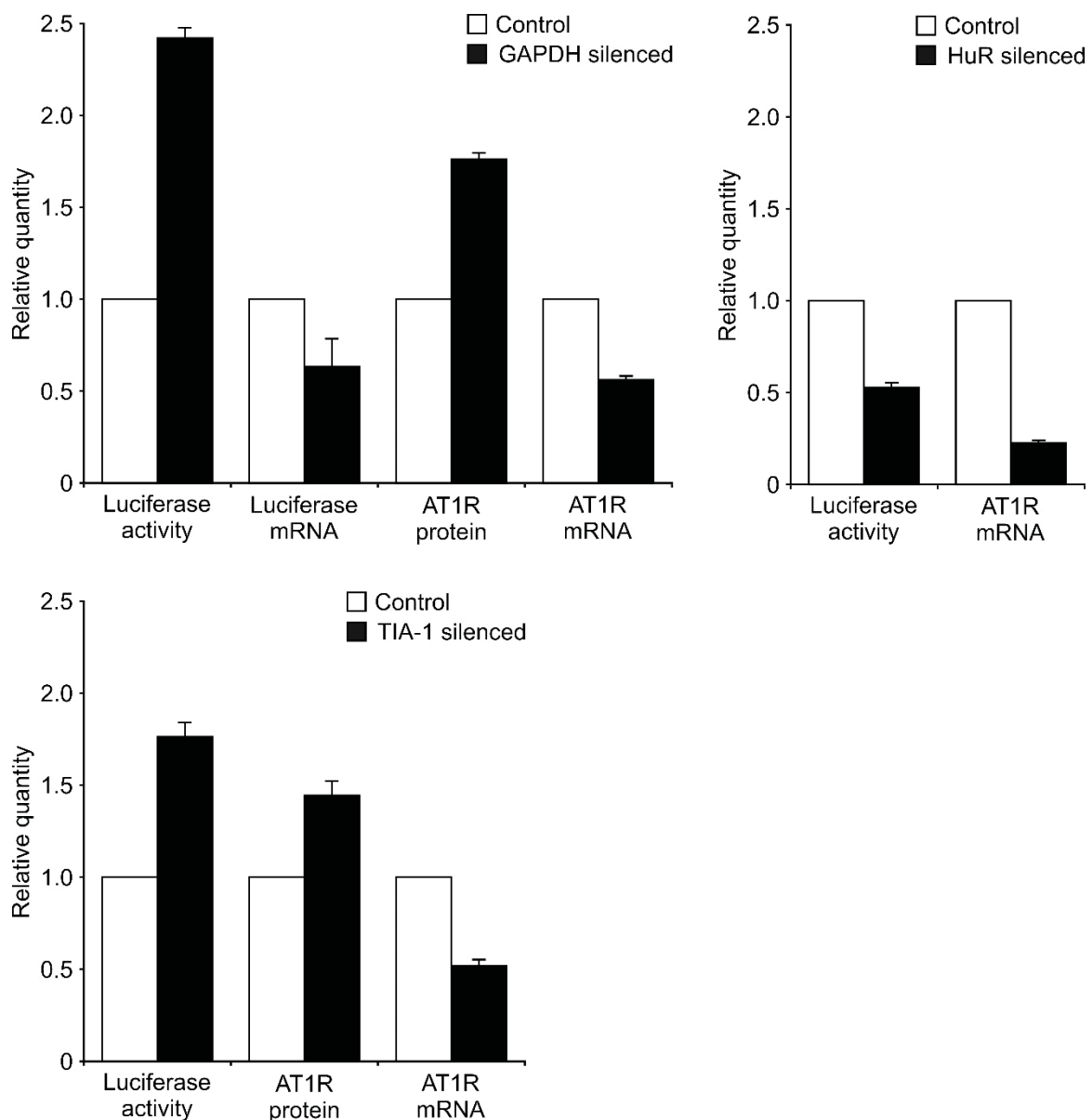
Figure 10. RNBP effects on AT1R expression. AT1R 3'UTR-binding RNBPs have been silenced as indicated. Luciferase protein and mRNA expression was studied in HEK293 transfected with an AT1R 3'UTR-bearing luciferase construct. Endogenous AT1R protein and mRNA levels were measured in VSMCs. The results are shown as mean ± SD of relative quantities. A combination of data from Studies I- III.

In Study II, the mechanisms underlying HuR-mediated AT1R regulation were explored. Silencing of HuR expression in HEK293 cells expressing a luciferase-reporter-gene construct decreased the luciferase expression in an AT1R 3'UTR-dependent manner. Similarly, HuR silencing reduced the endogenous AT1R mRNA in VSMCs **(Figure 10)**. In line with this, HuR overexpression had a positive effect on both protein and mRNA levels **(Study II, Figures 4B and 4C)**. This suggests that HuR upregulates AT1R expression by

affecting the mRNA expression. The mechanism was identified to be mediated via HuR-induced stabilization of the mRNA as HuR overexpression increased the half-life of a luciferase mRNA containing the AT1R 3'UTR, whereas silencing of HuR expression had an opposite effect **(Study II, Figure 5)**.

The regulatory mechanisms of TIA-1-mediated AT1R expression were investigated in Study III. TIA-1 silencing increased the protein expression of an AT1R 3'UTR-containing luciferase reporter construct in HEK293 cells and the endogenous AT1R protein expression in VSMCs. Conversely, endogenous AT1R mRNA levels were reduced in VSMCs following TIA-1 silencing **(Figure 10)**. Collectively, these results indicate that under normal conditions TIA-1 suppresses protein expression while stabilizing the mRNA. Further, both identified TIA-1 binding sites were independently capable of mediating the TIA-1 effect **(Study III, Figures 2B and 2C)**. However, as TIA-1 binding to the proximal region required GAPDH, inhibition of GAPDH binding, by using 3'UTR constructs lacking nucleotides 9 and 11, attenuated the TIA-1 effect at the proximal site **(Study III, Figure 3C)**. Neither TIA-1 nor GAPDH overexpression had any significant influence on the luciferase expression levels when GAPDH binding was blocked by this deletion construct.

4. Physiological and pathophysiological AT1R regulators and their RNBP mediators

The 3'UTR of AT1R mRNA destabilizes the mRNA under normal conditions as shown in Study I. Incorporation of the 3'UTR in a reporter gene construct leads to both decreased protein and mRNA expression. In studies I-III various environmental stimulations were shown to regulate the 3'UTR-mediated effects via the identified three RNBPs: GAPDH, HuR, and TIA-1.

In study I the effect of GAPDH on AT1R regulation was shown to be regulated by H_2O_2. Exposure of VSMCs to various concentrations of H_2O_2 increased AT1R expression in a dose-dependent manner, as shown by a western blot **(Study I, Figure 6A)**. The increase in AT1R expression was coupled to both decreased GAPDH expression and reduced association of GAPDH to AT1R 3'UTR **(Study I, Figures 6A and 6B)**. The diminished association of GAPDH to AT1R 3'UTR after H_2O_2 exposure was not only due to decreased

GAPDH expression, as treatment of VSMC lysates with H_2O_2 decreased the association without affecting GAPDH levels **(Study I, figure 6C)**.

The effect of GAPDH on H_2O_2-mediated regulation of AT1R was further studied by luciferase assay in HEK293 cells transfected with a control or AT1R 3'UTR containing luciferase reporter construct, together with a control or GAPDH expression vector. Subsequent exposure of the cells to H_2O_2 increased the luciferase expression when the AT1R 3'UTR was present. Simultaneous overexpression of GAPDH diminished the H_2O_2 effect validating the role of GAPDH as a mediator of the H_2O_2 response **(Figure 11)**. This was further confirmed by the observation that a luciferase construct lacking the nucleotides 9 and 11, needed for GAPDH binding, was unresponsive to both GAPDH overexpression and H_2O_2 as compared to controls **(Study I, Figure 6D)**.
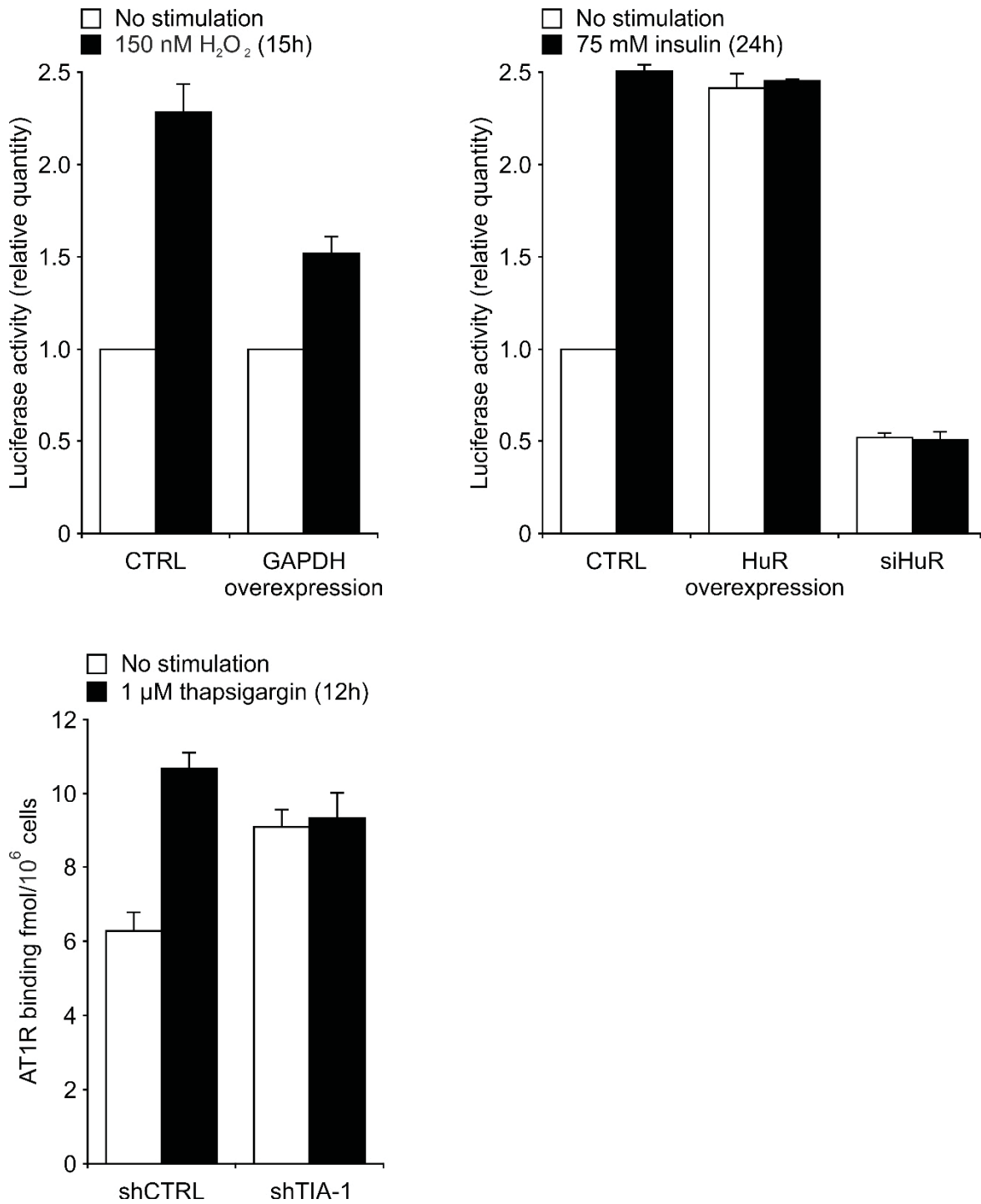
Figure 11. Factors regulating the effects of the identified GAPDH, HuR, and TIA-1 on AT1R expression. For H_2O_2 and insulin experiments, HEK293 cells were transfected with a luciferase expression construct bearing AT1R 3'UTR and the luciferase activity was measured to determine protein expression. In the thapsigargin experiment, the endogenous AT1R expression in VSMCs was measured by ligand-binding assay. The proteins of interest were overexpressed or silenced as indicated. The results are shown as mean ± SD. A combination of data from Studies I-III.

In Study II, insulin stimulation of VSMCs was shown to increase endogenous AT1R protein expression **(Study II, Figure 1A)**. In line with this, insulin increased AT1R mRNA expression in a dose-dependent manner by increasing the mRNA half-life **(Study II, Figures 1B and 1C)**. Insulin stimulation of HEK293 cells transfected with a luciferase reporter construct further demonstrated that the time-dependent increase in AT1R 3'UTR-mediated luciferase expression was coupled to increased nucleocytoplasmic HuR translocation and association with AT1R 3'UTR, as observed by affinity purification and REMSA **(Study II, Figures 3A-C)**.

The effect of insulin on AT1R regulation was shown to be mediated by HuR. HEK293 cells transfected with a luciferase reporter construct exhibited an AT1R 3'UTR-dependent increase in luciferase expression following insulin stimulation. Co-transfection with a HuR expression construct showed an increase in luciferase expression similar to that upon insulin stimulation, but no additional insulin effect was observed. Silencing HuR expression in turn decreased the luciferase expression and rendered it unresponsive to insulin **(Figure 11)**. In line with these results, a similar effect on endogenous AT1R protein and mRNA expression was observed in VSMCs where HuR was either overexpressed or silenced by lentiviral expression constructs prior to insulin stimulation **(Study II, Figure 4C)**. This shows that HuR is mediating the insulin effect on AT1R regulation.

Using luciferase constructs containing various AT1R 3'UTR fragments the insulin responsive element was identified to require at least the 637 first nucleotides of the 3'UTR. The first 607 nucleotides had only a minor effect on the reporter gene expression. This placed the insulin responsive element between nucleotides 607-637 **(Study 2, Figure 6B)**. Using the same luciferase constructs together with HuR overexpression, the HuR responsive element was shown to be the same as noted for insulin **(Study II, Figure 6C)**. This further supports the assumption that HuR serves as the mediator of the insulin response on AT1R expression,

As the function of HuR on posttranscriptional regulation of its targets requires its nucleocytoplasmic translocation (Fan and Steitz 1998a, Doller et al. 2008), the effect of blocking HuR translocation process on AT1R expression was studied. Silencing of HuR transporter CRM1 by shRNA or inhibition of its function by Leptomysin B (LMB) in

HEK293 cells, transfected with luciferase reporter gene construct, resulted in decreased responsiveness to insulin in a 3'UTR-dependent manner **(Study II, Figures 7A and 7B)**. As shown by western blotting, the effect was accompanied by inhibition of insulin-induced nucleocytoplasmic HuR translocation. In line with this, as studied by immunofluorescent microscopy, insulin stimulation increased cytoplasmic HuR accumulation in VSMCs whereas LMB inhibited the insulin-induced nuclear export of HuR **(Study II, Figure 7C)**.

In study III, the TIA-1-mediated effects on AT1R were observed to be regulated by ER stress. Ligand-binding experiments revealed that thapsigargin-induced ER stress increased the AT1R protein expression in VSMCs. Silencing TIA-1 expression by lentiviral shRNA constructs led to increased baseline AT1R expression but no additional effect was seen after thapsigargin exposure. **(Figure 11)**. Thapsigargin treatment induced the expression of various stress marker proteins and reduced the total mRNA expression levels while the relative AT1R mRNA expression was increased **(Figure 12)**. Endogenous mRNA levels also showed no significant response to thapsigargin in TIA-1-silenced VSMCs compared to controls. In unstimulated cells however, TIA-1 silencing decreased the relative baseline AT1R mRNA levels, in line with previous results **(Study III, Figure 5B)**. This indicates that TIA-1 suppresses AT1R expression under normal conditions and that the suppression is attenuated by ER stress.
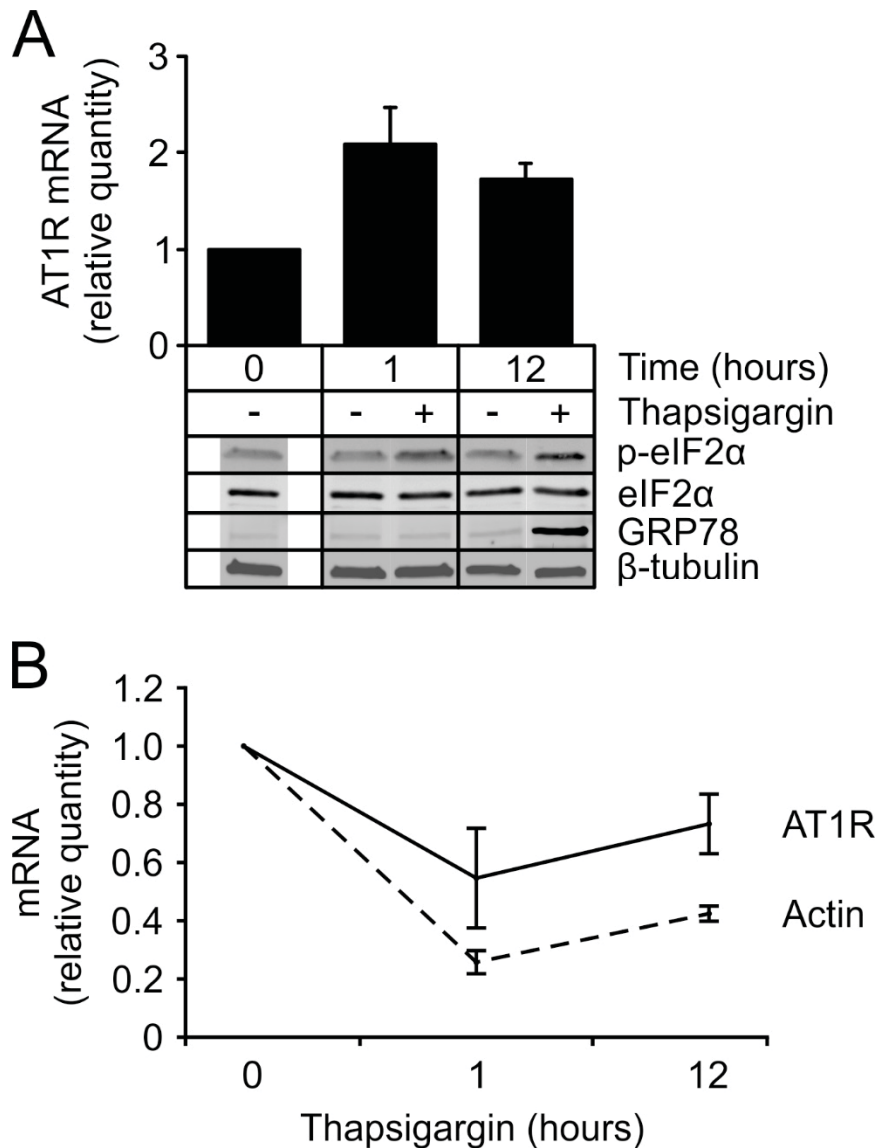
Figure 12. Thapsigargin effect on AT1R mRNA expression. **(A)** Thapsigargin-induced increase in stress marker proteins and relative AT1R mRNA expression. A combination of data from Study III. **(B)** Thapsigargin effect on total mRNA levels of AT1R and actin that was used for the normalization of the results (unpublished data). The results are shown as mean ± SD of relative quantity to results at 0 hours.

Using immunofluorescent microscopy and fluorescent *in situ* hybridization, both TIA-1 and GAPDH were shown to colocalize with AT1R mRNA in VSMCs under normal conditions. However, thapsigargin-induced ER stress dissociated TIA-1 from AT1R mRNA. During ER stress, TIA-1 translocated to SGs, leaving AT1R mRNA to the cytoplasm. Association of GAPDH to AT1R mRNA was unaffected by ER stress while a fraction of GAPDH localized to SGs as well **(Figure 13)**.
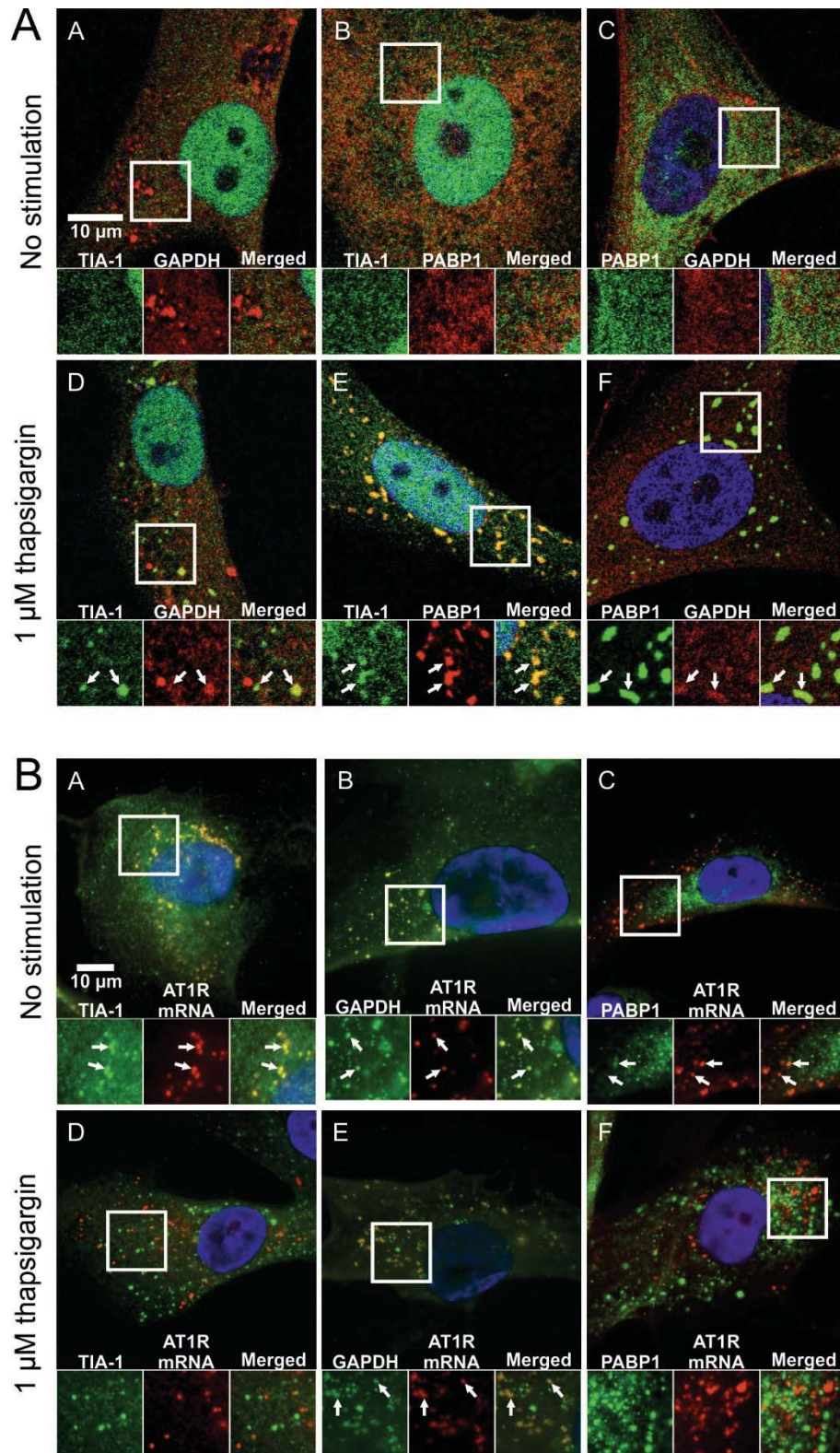
Figure 13. Effect of ER stress on TIA-1 or GAPDH cellular localization and association to AT1R mRNA. **(A)** Localization of TIA-1, GAPDH, and a SG-marker protein PABP in unstressed VSMCs (A-C) or in VSMCs under ER stress (D-F). **(B)** Association of TIA-1, GAPDH, or PABP to AT1R mRNA in unstressed VSMCs (A-C) or in VSMCs under ER stress (D-F). The arrows show colocalization of the signals. A Combination of data from Study III.

The results from Studies I-III with respect to the identified AT1R 3'UTR *trans*-acting factors, their various effects and modes of action are summarized in **Table 3**.

Table 3. Summary of the AT1R 3'UTR *trans*-acting factors and their regulatory mechanisms. ND = not determined.

ARE-BP	Effect	Mechanism	Experimental model	Region (3'UTR)	Binding to mRNA	Study
GAPDH	Negative	Translation & stability	H_2O_2 dissociates GAPDH from 3'UTR	1-100	Direct	I
HuR	Positive	Stability	Insulin increases HuR association to 3'UTR	607-637	ND	II
TIA-1	Negative	Translation & stability	Thapsigargin dissociates TIA-1 from 3'UTR	1-100 600-887	Via GAPDH (1-100) & direct (600-887)	III

DISCUSSION

1. AT1R 3'UTR *trans*-acting factors

1.1 Initial studies

The present series of investigations were set up to identify novel AT1R 3'UTR *trans*-acting factors involved in the regulation of AT1R expression. The first indications of 3'UTR-mediated regulation of AT1R mRNA were made by Bonnardeaux et al. (1994) when they characterized a 3'UTR-located A1166C SNP that seemed to be associated with essential hypertension. Their study suggested that the identified SNP might function as a marker for an unidentified functional variant. Later this SNP was shown to overlap with a miR-155 binding site. The CC genotype was further shown to inhibit miR-155 binding and to be associated with both elevated blood pressure and AT1R protein expression (Ceolotto et al. 2011). Since the first discovery of the A1166C SNP, several other studies focusing on the regulation of AT1R expression on mRNA level suggested a posttranscriptional model orchestrated by RNBPs (Lassègue et al. 1995, Nickenig and Murphy 1996). Finally AUF-1 was identified as such a posttranscriptional regulator (Pende et al. 1999). Since then, several other proteins and miRNAs have also been identified, implicating the 3'UTR as one of the key regulatory targets of AT1R expression (Nickenig et al. 2002, Paukku et al. 2008, Sansom et al. 2010, Guo et al. 2015). In this thesis, three new RNBPs have been characterized as 3'UTR-mediated posttranscriptional regulators of AT1R expression. The currently known AT1R 3'UTR *trans*-acting factors and their binding sites are shown in **Figure 14**.
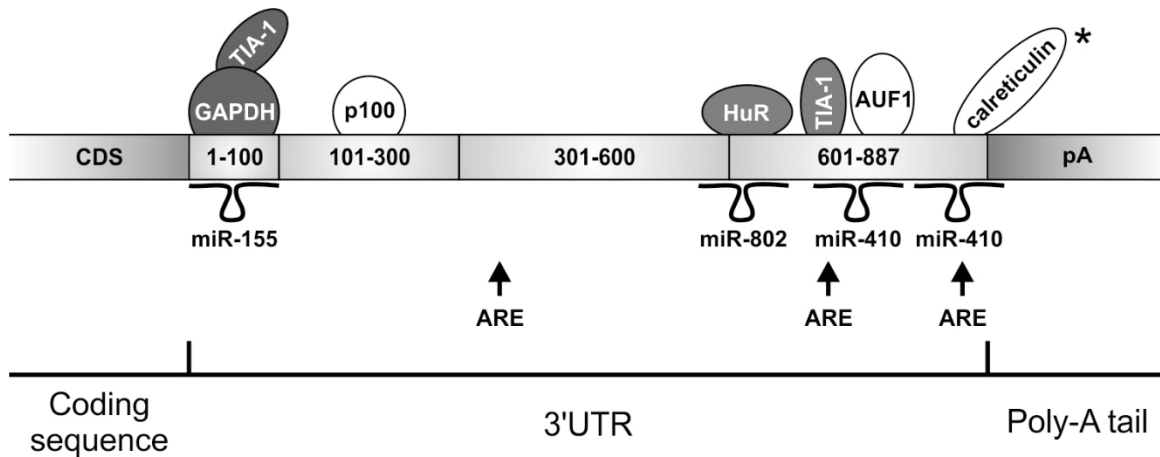
Figure 14. A schematic representation of known AT1R 3'UTR *trans*-acting factors and their binding sites. Locations of AREs are shown (arrows). *, the binding site is based on experiments performed in the rat and no details are available in humans.

1.2 GAPDH

GAPDH was shown to associate with the 3'UTR of AT1R mRNA, increasing the mRNA stability while suppressing its translation under normal conditions (Study I). When exposed to an oxidizing environment, however, GAPDH was released from the mRNA leading to increased AT1R protein expression. The dissociation of GAPDH from AT1R mRNA in response to H_2O_2 treatment, was coupled with overall reduction of GAPDH expression. Rodriguez-Pascual et al. (2008) and Kondo et al. (2011) identified GAPDH as a redox-sensitive regulator of mRNA expression. A similar mechanism has been proposed in the present study where H_2O_2 abolishes GAPDH-AT1R 3'UTR interaction. H_2O_2 is known to reduce both GAPDH half-life (Sukhanov et al. 2006) and activity (Janero et al. 1994, Schuppe-Koistinen et al. 1994, Ciolino and Levine 1997). This is in line with the present study showing that H_2O_2 induces reduction of both GAPDH expression and its association to AT1R 3'UTR. It should be noted that H_2O_2 increases both eNOS expression and enzymatic activity, thus increasing NO production (Drummond et al. 2000, Thomas et al. 2002). In addition to H_2O_2, NO is a known inhibitor of GAPDH by nitrosylation of its active site (McDonald and Moss 1993, Padgett and Whorton 1995). Other oxidation-induced modifications such as aggregation of GAPDH (Nakajima et al. 2007) or protein-protein interactions (Kim et al. 2003) induced by H_2O_2, should also be considered as possible mechanisms by which H_2O_2 exposure may lead to dissociation of GAPDH from AT1R mRNA.

While the function of GAPDH in SG assembly has not been described in detail, the present study demonstrates partial localization of GAPDH to SGs after thapsigargin-induced ER stress (Study III). This is supported by another study where GAPDH was identified as an SG component when searching for SG components after arsenite-induced SG assembly (Ohn et al. 2008). In that study, the role of GAPDH in SGs was not described in more detail, however. The present thesis further supports the localization of GAPDH to SGs but its exact physiological role in these organelles remains to be further explored.

1.3 HuR

In the present study HuR was shown to mediate the insulin-induced upregulation of AT1R protein expression via a posttranscriptional mechanism. HuR associates with the AT1R 3'UTR leading to increased mRNA stability, the effect of which is increased by insulin (Study II).

The relationship between insulin and AT1R has been established previously. In a study by Nickenig et al. (1998), insulin increased AT1R expression in rat VSMCs, and the underlying mechanism was reported to involve posttranscriptional stabilization of the AT1R mRNA. In the present study, the insulin responsive element in human AT1R mRNA was mapped to the 3'UTR. A luciferase reporter construct containing the AT1R 3'UTR showed increased mRNA and protein expression in response to insulin stimulation.

When searching for proteins mediating the insulin effect via the AT1R 3'UTR, HuR was identified as a candidate. HuR is one of the best characterized ARE-BPs. As such, it was not unexpected to find it associated with the AT1R 3'UTR bearing multiple AREs (Study II). In fact, Pende et al. (2008) were the first to suggest a possible role of HuR in posttranscriptional regulation of AT1R. They demonstrated that Ang II induced the nucleocytoplasmic translocation of a HuR fusion protein, and a purified HuR fusion protein was shown to bind AT1R 3'UTR. The mechanisms or effects of HuR on AT1R expression were not studied in more detail by Pende et al. (2008). The present study further expands these original observations. In fact, it was confirmed that endogenous HuR was able to bind to the AT1R 3'UTR leading to increased stability of the mRNA (Study II). This is in line with the best known actions of HuR as a stabilizing factor of its target transcripts.

One of the prerequisites for HuR action is its nucleocytoplasmic translocation. This is consistent with the results from the present study in which insulin induced this translocation. A transporter protein CRM-1 was shown to mediate HuR translocation and to be required for the insulin-induced upregulation of a luciferase reporter construct bearing AT1R 3'UTR. Inhibition of CRM1 by siRNA, or by a specific blocker (LMB), inhibited the cytoplasmic HuR translocation and attenuated the insulin effect on luciferase expression. This suggests that the insulin-induced upregulation of AT1R expression by HuR require the nucleocytoplasmic translocation of HuR by CRM1.

In the study by Nickenig et al. (1998), insulin-mediated upregulation of AT1R expression was identified as MAPK-dependent. Another study by Subbaramaiah et al. (2003) reported that stabilization of Cox-2 mRNA by HuR was dependent on p38 MAPK and MAPK-activated protein kinase 2 (MK2). Insulin is known to both activate p38 MAPK and induce the nucleocytoplasmic translocation of CRM1 (Begum and Ragolia 2000, Somwar et al. 2000, Kim et al. 2011). In addition, a study by Gurgis et al. (2015) reported that increased IL-6 mRNA stability was dependent on the p38 MAPK-MK2-HuR pathway. Taken together, these previous observations combined with the results from Study II, describing insulin-induced nucleocytoplasmic translocation of HuR by CRM-1, paint a broad outline of a possible signaling cascade required for the insulin-induced stabilization of AT1R mRNA by HuR **(Figure 15)**. However, this signaling cascade and its detailed mechanisms need to be confirmed.
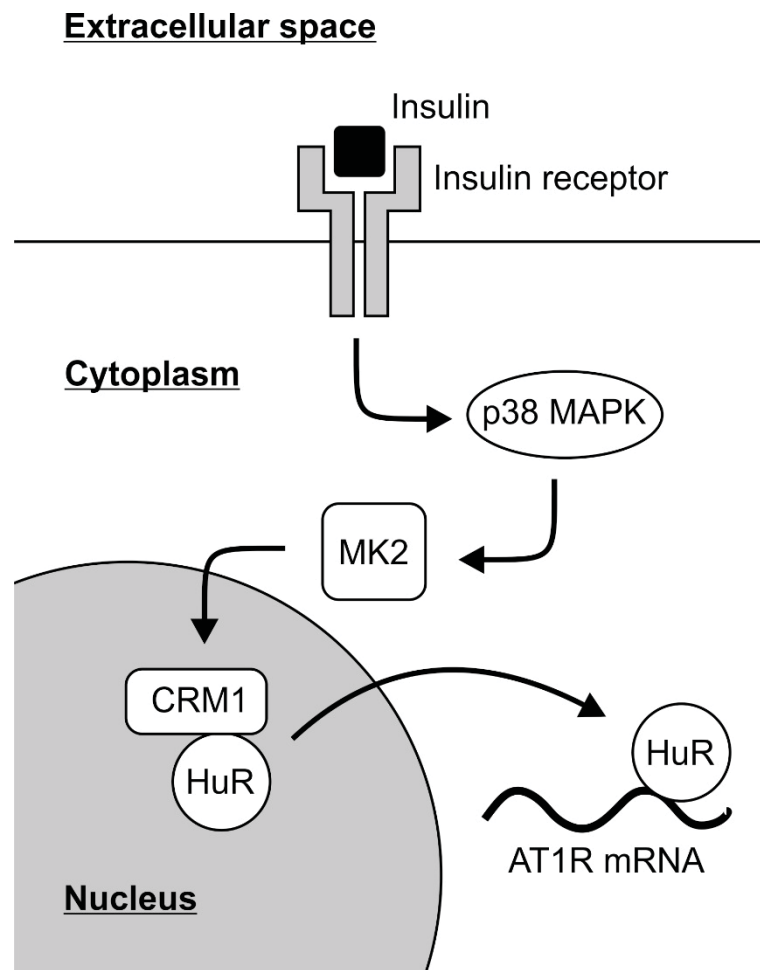
Figure 15. Outline of a possible signaling cascade resulting in insulin-induced association of HuR with AT1R mRNA

1.4 TIA-1

TIA-1 was shown to bind to both the proximal and distal regions of the AT1R 3'UTR (Study III). The association at the proximal site was identified to be GAPDH-dependent but binding at the distal site was direct and independent of other proteins. Three different mechanisms for interaction may be proposed **(Figure 16)**. In model A the two TIA-1 binding sites are independent and do not interact with each other, whereas in model B homodimerization of TIA-1 from the two sites is proposed to cause a loop-structure in the mRNA. The homodimerization of TIA-1 is supported by a study describing TIA-1 aggregation via its prion-related domain in SG assembly (Gilks et al. 2004). Looping of the mRNA would enable protein-protein or protein-RNA interactions over distinct regions. The H_2O_2-induced dissociation of GAPDH from the AT1R mRNA (Study I) would dissociate TIA-1 from the

proximal site suggesting that this site might be involved in the ROS sensitive regulation via TIA-1 and GAPDH. In model B, GAPDH and TIA-1 dissociation from the proximal site would further attenuate the conformational change of the mRNA molecule by disrupting the loop structure. TIA-1 binding at the distal site is not GAPDH-dependent and the TIA-1 effects at this site may be controlled by some other mechanisms.
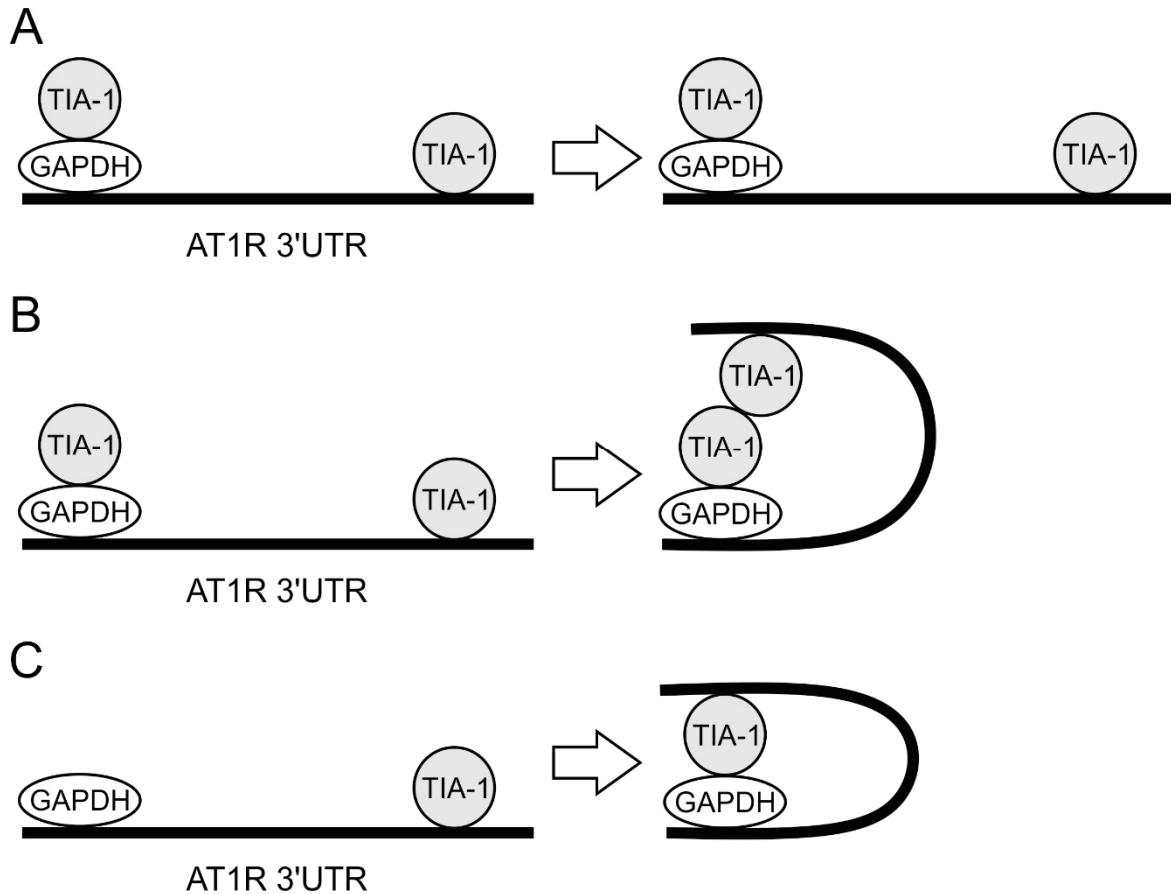
Figure 16. Proposal of models for GAPDH-TIA-1 interactions with AT1R 3'UTR

In model C, TIA-1 would only bind to the distal region and the proximal association (Study III) would be due to protein-protein interactions of TIA-1 and GAPDH over the distinct binding sites. This is supported by a previous study showing protein-protein interactions between TIA-1 and a small nuclear ribonucleoprotein (snRNP) in spliceosome assembly (Förch et al. 2002). Also in model C, GAPDH and TIA-1 heterodimerization would cause a loop structure in the mRNA. Dissociation of either of the proteins would lead to disruption of the loop, leaving the other protein at its place in the mRNA.

The best described role of TIA-1-induced translational suppression involves the recruitment of target mRNAs to SGs during cellular stress. However, AT1R mRNA was shown to be suppressed by TIA-1 under normal conditions but escape recruitment to SGs by dissociation of TIA-1 (Study III). The mechanism of TIA-1-mediated regulation of its target mRNAs in unstressed environment is poorly known but it may involve regulation of transcript variants via splicing or translational suppression and mRNA decay (Izquierdo and Valcárcel 2007, Yamasaki et al. 2007). Further, the 3'UTR is known to be involved in translational regulation of some mRNAs. While the detailed mechanisms are still poorly known, they involve protein-protein interactions over the 5' and 3'UTRs by looping of the mRNA and affecting the assembly of the translation initiation complexes (Szostak and Gebauer 2013). The TIA-1-mediated conformational changes in AT1R mRNA via protein-protein interactions, proposed in Figure 16 models B and C, would support this mechanism but this remains to be confirmed.

2. AT1R as a stress-regulated receptor

The expression of AT1R has been shown to be increased under various conditions associated with both the development of CVDs and cellular stress. In this thesis, three models of such stressful environments and their affected RNBPs were explored: oxidative stress via GAPDH (Study I), hyperinsulinemia via HuR (Study II), and ER stress via TIA-1 (Study III).

2.1. AT1R and cellular stress

H_2O_2 increases the ROS load in the cells, exposing them to oxidative stress. The association between ROS and AT1R expression is well known. While AT1R activation increases ROS production, AT1R expression and function have been shown to be increased by ROS (Li et al. 2000, Nickenig and Harrison 2002, Sungkaworn et al. 2013, Bhatt et al. 2014). This may lead to a vicious cycle accelerating the cellular oxidative stress. However, the molecular mechanisms involved in ROS-dependent upregulation of AT1R expression are poorly known. In Study I, H_2O_2 increased AT1R protein expression by releasing the translational suppressor GAPDH from AT1R 3'UTR. Bhatt et al. (2014) further described AT1R

upregulation by H_2O_2 to involve activation of transcription factor NF-κB. Interestingly, NF-κB activation is also induced by AT1R activation (Kranzhöfer et al. 1999, Mitra et al. 2010, Young et al. 2015). This would enable a positive feedback loop promoting AT1R upregulation. Taken together, these results shed light on how AT1R expression may be upregulated by ROS via both transcriptional and posttranscriptional mechanisms.

In Study II, the effect of insulin on posttranscriptional regulation of AT1R expression was explored. Type 2 diabetes (T2D) is a common risk factor for CVD and is often associated with hyperinsulinemia. Previous studies have established the link between T2D, insulin, and AT1R expression. In atherosclerotic arteries of a diabetic mouse model, AT1R expression is increased compared to non-diabetic arteries (Ihara et al. 2007). Further, in human carotid artery samples from T2D patients, AT1R expression is increased compared to non-diabetic patients. In VSMC cultures from these samples, similar baseline increase in AT1R expression and insulin-induced increase in both AT1R mRNA and protein levels may be observed (Hodroj et al. 2007). In the study by Nickenig et al. (1998), insulin was shown to increase the relative AT1R mRNA levels in both a time and dose-dependent manner and the results were coupled to increased expression of the membrane-bound receptor. While insulin had no effect on the transcription rate of *AT1R* gene, it increased the half-life of the mRNA. Thus, a posttranscriptional regulatory mechanism was proposed (Nickenig et al. 1998). This is in accordance with the results of the present study in which the positive insulin effect on AT1R expression was mediated via the 3'UTR of AT1R mRNA (Study II). Insulin increased cytoplasmic HuR accumulation and binding to AT1R 3'UTR. Insulin-mediated binding of HuR to AT1R 3'UTR was further shown to stabilize the mRNA and increase protein expression. While the functionality of this posttranscriptional regulatory mechanism remains to be confirmed *in vivo,* it proposes an elegant model of how AT1R expression may be regulated by insulin.

The fact that both ROS and insulin increase AT1R expression, and that both are known ER stressors, raises the question of whether AT1R is a general ER-stress-regulated protein. Thapsigargin induces ER stress by increasing the cytoplasmic free Ca^{2+} levels via inhibition of the SERCA family of Ca^{2+} pumps (Thastrup et al. 1990, Lytton et al. 1991). In this thesis, the effect of ER stress on AT1R expression was explored using thapsigargin. AT1R mRNA was shown to escape thapsigargin-induced sequestration into SGs (Study III). While it needs

to be confirmed, this would allow AT1R mRNA to remain accessible for the translational machinery during ER stress. This is supported by the relative increase in both AT1R mRNA and protein levels during ER stress in VSMCs. This is in line with a previous study describing that AT1R mRNA remains translationally active under cellular stress (Martin et al. 2003). In addition to posttranscriptional mechanisms, AT1R expression may be upregulated under cellular stress via NF-κB-mediated transcriptional control. A number of studies show that NF-κB is activated by oxidative and ER stress (Pahl and Baeuerle 1995, Pahl and Baeuerle 1996, Deng et al. 2004). This suggests that both transcriptional and posttranscriptional mechanisms regulate AT1R expression during cellular stress.

While ER stress may be caused by several mechanisms it is not known if they all have similar impact on AT1R expression. H_2O_2-induced oxidative stress was shown to dissociate GAPDH from AT1R mRNA (Study I) while during thapsigargin-induced ER stress TIA-1 was dissociated but GAPDH remained colocalized with AT1R mRNA (Study III). At the same time, a fraction of GAPDH localized to SGs. This suggests that a different pool of GAPDH localizes to SGs than that associating with AT1R mRNA. Whether this is due to some posttranslational modification of GAPDH remains to be answered. Different cellular stressors may also have different effects on the mRNA-binding capabilities of GAPDH.

Phosphorylation of eIF2α is considered to be a proxy for ER stress and a prerequisite to SG formation. However, various stressors phosphorylate eIF2α via different kinases (Donnelly et al. 2013). In addition, the mRNA subsets affected by phosphorylation of eIF2α have been shown to differ depending on the kinase phosphorylating eIF2α (Dang Do et al. 2009). Emara et al. (2012) proposed that H_2O_2-induced SGs differ from the canonical SGs and are assembled independently of eIF2α phosphorylation. Further, Larroque-Cardoso et al. (2013) showed that in human VSMCs, oxLDL is capable of inducing eIF2α phosphorylation but require PKCδ for activation of pro-apoptotic pathways, whereas thapsigargin is not dependent on PKCδ and can also activate the apoptotic pathway in PKCδ deficient cells. Taken together, the ER stress pathways are diverse and complex, suggesting that the stress-induced regulation of AT1R expression may differ depending on the stressor.

The cellular stressors used in this thesis and the mechanism by which they increase AT1R expression via GAPDH, HuR, and TIA-1 are summarized in **Figure 17.**
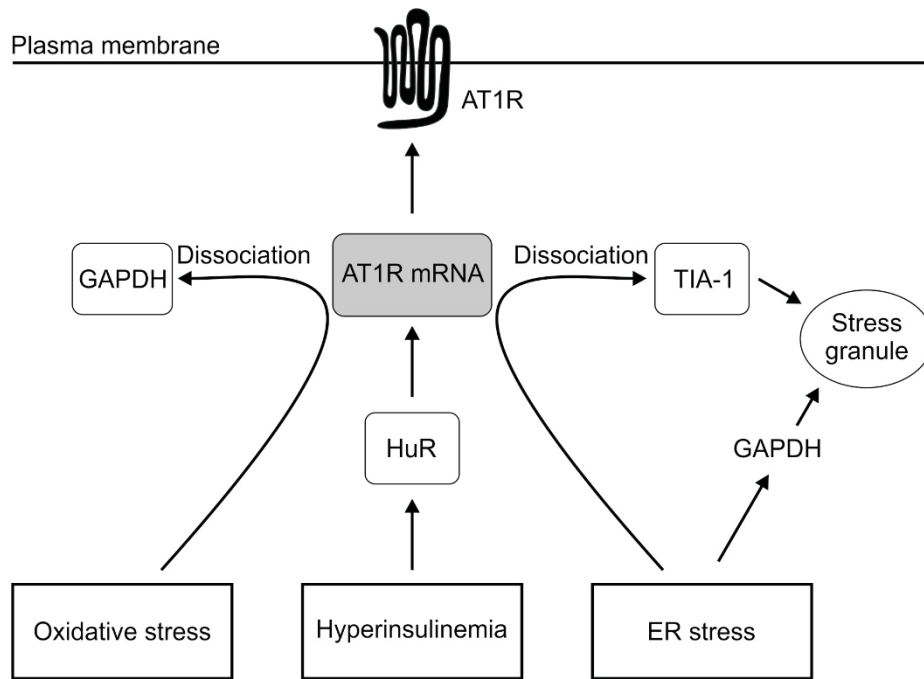
Figure 17. A summary of the mechanisms by which oxidative stress, hyperinsulinemia, and ER stress increase AT1R expression by affecting GAPDH, HuR, and TIA-1 binding to the 3'UTR of AT1R mRNA.

2.2. GAPDH, HuR and TIA-1 as mediators of stress responses

The RNBPs identified in this thesis, i.e. GAPDH, HuR, and TIA-1, have been previously associated to various stress response pathways. All of them have been shown to posttranscriptionally regulate the expression of Cox-2, a central mediator of inflammatory responses (Dixon et al. 2003, Cok et al. 2003, Ikeda et al. 2012). Cox-2 is expressed in fatty streaks in arteries from both human and mice, and Cox-2 mRNA is upregulated in atherosclerotic plaques (Cipollone et al. 2004, Baldan et al. 2014). Another inflammatory cytokine, TNFα, is also posttranscriptionally regulated by GAPDH, HuR, and TIA-1 (Piecyk 2000, Dean et al. 2001, White et al. 2015). A number of CVD risk factors are associated with cellular events that have been proposed to involve TNFα. These include cardiac remodeling (Zhao et al. 2008), vascular calcification (Masuda et al. 2013), inflammation (Li et al. 2005), and AT1R upregulation (Gurantz et al. 1999, Cowling et al. 2002). These studies indicated that ER-stress-related pathways were involved in mediating TNFα effects. Further, HuR has been shown to promote, and TIA-1 to inhibit, the translation of apoptosis regulator, mitochondrial cytochrome c. The regulation is affected by ER stress that dissociates HuR from the mRNA, leaving it under the suppressive control of TIA-1

(Kawai et al. 2006). Taken together, in addition to the posttranscriptional regulation of AT1R expression described in this thesis, GAPDH, HuR, and TIA-1 regulate a number of other factors associated with the development of CVDs. All in all, this suggests that GAPDH, HuR, and TIA-1 may form a posttranscriptional regulatory complex regulating several components of the CVD predisposing pathways.

3. Challenges in studying the regulation of AT1R expression

Currently the most common methods to study AT1R expression include measurement of AT1R mRNA expression by qPCR, measurement of the activation of downstream signaling molecules in response to Ang II, immunological assays using antibodies against AT1R, and measurement of membrane-expressed receptor by ligand-binding assay using radiolabeled Ang II. Of these, only the ligand-binding assay and immunological methods indicate actual changes in expression at the level of receptor protein. The changes in mRNA expression as measured by qPCR do not always correlate with changes in the protein expression. Changes in expression or activation of AT1R downstream signaling molecules in response to Ang II stimulation do not alone confirm alterations in AT1R expression. In contrast, they may simply indicate changes in AT1R signaling due to alteration in the balance of transcription variants or dimerization of the receptor (Warnecke et al. 1999, Martin et al. 2001, Martin et al. 2006, AbdAlla et al. 2000, AbdAlla et al. 2001a, AbdAlla et al. 2001b, Sungkaworn et al. 2013). While immunological methods are generally considered to be an established way to demonstrate actual changes at the protein level, they have proved to be challenging in the assessment of AT1R. Recent studies describe lack of specificity of the commercially available AT1R antibodies (Benicky et al. 2012, Herrera et al. 2013, Elliott et al. 2013). For this reason there is a need to generate more specific antibodies: either monoclonal or peptide antibodies. For the time being, ligand-binding assays may offer the most reliable approach to quantitate AT1R protein expression levels.

In the current study, the proteins associated with AT1R 3'UTR were isolated by affinity purification and separated by SDS-PAGE. Thereafter the gels were either silver or coomassie-blue stained, the bands of interest were cut out, the proteins were treated with trypsin to generate peptides and identified by mass spectrometry. While this is a relevant

method, some AT1R 3'UTR-associated proteins may have escaped detection. Affinity purification relies on synthetic RNA probes consisting of only the 3'UTR. This may have an impact on the formation of the RNA secondary structures, like looping, affecting protein binding. Moreover, the silver and coomassie blue stainings are adequate techniques to detect abundantly-associated proteins, whereas proteins binding to lesser extent or only under specific conditions may remain undetected even when using a mass spectrometry approach.

The use of GAPDH as a loading control or housekeeping gene in experimental setups should be considered carefully, especially in experiments using oxidative conditions. As shown in Study I, H_2O_2 decreased GAPDH expression. This is in line with a previous study showing reduced half-life of GAPDH due to H_2O_2-mediated oxidation (Sukhanov et al. 2006). In addition, the use of GAPDH for normalization of data has been questioned before in a study showing increased GAPDH mRNA expression during atherogenesis (Hiltunen et al. 1995). These observations combined with the multiple known functions of GAPDH in various cellular events makes its use as an experimental control questionable. If GAPDH expression is affected, the results will be incorrect when normalized against it. Therefore the use of multiple controls or total protein staining for western blots should be considered for more reliable normalization of data (Vandesompele et al. 2002, de Jonge et al. 2007, Gürtler et al. 2013, Li and Shen 2013).

An additional technical caveat may come from recent studies proposing that AT1R mRNA expression may be differentially regulated in different cell types or tissues. An insulin-resistant rat model with increased plasma insulin levels showed increased AT1R expression in thoracic arteries, in line with the results from Study II. However, the AT1R expression in the abdominal arteries was unchanged, suggesting that the regulatory mechanisms of AT1R may differ even in closely-connected areas (Karpe et al. 2012). In another study by Gao et al. (2014), AT1R mRNA localized into SGs in HeLa cells, which is opposite to observations from Study III where AT1R mRNA escaped sequestration to SGs in VSMCs. This suggests that AT1R regulation may be cell-type specific. This also raises the question of using immortalized cell lines as experimental models. While they are easy to work with, they differ from normal diploid cells significantly. Most of the immortalized cell lines have severe changes in their karyotypes (www.atcc.org/). As such, the results should be confirmed in primary cells if possible. It should be noted, however, that primary cells may

also have genetic and epigenetic differences, depending on the patients they have been obtained from.

CONCLUSIONS

Cardiovascular diseases (CVDs), including hypertension and atherosclerosis, constitute the leading cause of death and disability globally. CVDs are multifactorial diseases and several underlying mechanisms contribute to the risk of their development. AT1R is a central component of the renin-angiotensin system regulating the blood pressure as well as body fluid and electrolyte homeostasis. AT1R signaling also results in the generation of ROS that contribute to various deleterious cellular effects in CVDs. The role of AT1R in the development of CVDs is supported by the beneficial effects achieved by pharmacological inhibition of AT1R function.

AT1R expression is regulated at multiple levels but the underlying mechanisms are not completely understood. The aim of this thesis was to identify novel RNBPs involved in the posttranscriptional regulation of AT1R expression. The 3'UTR of AT1R mRNA has emerged as a key regulatory element that posttranscriptionally regulates AT1R expression via *trans*-acting factors. During the course of this study, three novel RNBPs regulating AT1R expression via AT1R 3'UTR were identified: GAPDH, HuR, and TIA-1. While certain aspects of the individual roles of these three *trans*-acting factors posttranscriptionally regulating AT1R mRNA expression have been delineated in the present thesis, the details of their cooperation and interactions remain to be resolved.

Under normal physiological conditions, both GAPDH and TIA-1 repress AT1R expression while HuR increases it. GAPDH inhibits the translation of AT1R mRNA while stabilizing the mRNA transcript. Similarly, TIA-1 suppress AT1R protein expression while having a positive effect on the mRNA levels. On the other hand, HuR stabilizes the AT1R mRNA leading to increased AT1R protein expression. Together with previous studies, these findings further strengthen the role of AT1R 3'UTR as an important regulatory element in AT1R expression. The 3'UTR of AT1R is a target for a number of both positive and negative *trans*-acting factors. This proposes that the expression of AT1R may be controlled by affecting the balance between the positive and negative regulators associated with the 3'UTR.

The expression of AT1R is affected by various pathophysiological stimuli by regulating the association of *trans*-acting factors to the 3'UTR. Under oxidizing conditions, overall GAPDH expression is reduced and GAPDH dissociated from the AT1R mRNA. This releases AT1R mRNA from the translational suppression of GAPDH leading to increased AT1R protein expression. The insulin-induced upregulation of AT1R expression is mediated by HuR via AT1R 3'UTR. Insulin stimulation increases the nucleocytoplasmic translocation of HuR. This is accompanied by increased association of HuR with AT1R 3'UTR, promoting the positive impact of HuR on AT1R expression. During ER stress, caused by ER Ca^{2+} deprivation, AT1R mRNA escapes the sequestration to translationally silenced SGs. ER stress releases the SG component TIA-1 from AT1R mRNA, whereafter unbound TIA-1 is directed to SGs releasing AT1R mRNA from TIA-1 suppression. As a result, the relative AT1R protein expression is increased during ER stress.

Taken together, the results of this thesis suggest that insulin resistance, ROS, and ER stress may contribute to the development of CVDs by affecting posttrancriptional regulation of AT1R expression. Better understanding of the complex network of posttranscriptional regulation of AT1R expression may create new possibilities to assist in the investigation of the pathophysiology and novel treatments of human CVDs.

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