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Transgenic animal models for the functional analysis of vasoactive peptides

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Abstract

The interplay of vasoactive peptide systems is an essential determinant of blood pressure regulation in mammals. While the endothelin and the renin-angiotensin systems raise blood pressure by inducing vasoconstriction and sodium retention, the kallikrein-kinin and the natriuretic-peptide systems reduce arterial pressure by eliciting vasodilatation and natriuresis. Transgenic technology has proven to be very useful for the functional analysis of vasoactive peptide systems. As an outstanding example, transgenic rats overexpressing the mouse *Ren*-2 renin gene in several tissues become extremely hypertensive. Several other transgenic rat and mouse strains with genetic modifications of components of the renin-angiotensin system have been developed in the past decade. Moreover, in recent years gene-targeting technology was employed to produce mouse strains lacking these proteins. The established animal models as well as the main insights gained by their analysis are summarized in this review.

Key words
- Renin-angiotensin system
- Transgenic
- Animal models
- Knockout

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Introduction

Peptides such as angiotensins, kinins, endothelins, vasopressin and natriuretic peptides are the most important regulators in the cardiovascular system. They are liberated from larger precursor proteins by metabolizing enzymes and exert their actions by binding to receptors belonging to the class of heptahelical membrane proteins coupled to G-proteins (except the natriuretic-peptide receptors, which represent membrane-bound guanylate cyclases). These peptide systems were originally thought to act mainly in an endocrine manner since in most cases the precursor proteins and the processing enzymes are produced in distinct tissues and assemble in the circulation to produce the active peptides, which interact with receptors in cardiovascular target organs like heart, vessel wall, and kidney. However, in recent years the local production of peptides by precursors and enzymes jointly synthesized in several tissues has been detected and important regulatory functions have been ascribed to such tissue peptide systems. Especially in the case of short-lived peptides like kinins and endothelins this functioning mode seems to be more relevant than the endocrine action as much higher concentrations of the peptides can be reached at the receptor when they are produced in its immediate neighborhood. The functions of the locally produced peptides may be largely independent of the circulating systems especially at sites to which the circulating pep-

tides have no access under normal circumstances, e.g., beyond the blood-brain and blood-testis barriers.

While the circulating peptide systems can be successfully studied using inhibitors of the processing enzymes or receptor antagonists, for the functional analysis of local peptide systems such pharmacological methods are inappropriate because they lack tissue specificity. The injection of specific antibodies or antisense oligonucleotides against components of peptide systems in tissue exerts more local effects. However, these procedures are hampered by low efficiency, a short duration of action and side effects elicited by the injection procedure itself. Therefore, transgenic techniques have been increasingly applied for the functional analysis of local peptide systems. Transgenic techniques will be briefly described in the following sections and the suitability of the transgenic approach will be illustrated by the description of transgenic animal models with targeted modifications in the renin-angiotensin system.

Transgenic technology

Two different approaches can be used to modify the genotype of an animal: 1) the microinjection of DNA in the nucleus of a fertilized oocyte, and 2) the targeted modification of a gene in its chromosomal location by homologous recombination in embryonic stem cells, which are subsequently integrated into early embryos. In both cases the modified embryos develop into transgenic animals after transfer into foster mothers.

Microinjection technique

The technique for the production of transgenic animal models by the integration of foreign gene sequences into the genome of mammals has been established for more than 15 years (1-4). Most of the experiments since then have been performed on mice (reviewed in 5-8). However, the technique has also been extended to species like rats (9-12), rabbits (13,14), sheep (14,15), goats (16), cattle (17), and pigs (13,14,18-20). The development of transgenic technology for the rat was especially important for cardiovascular research, in which the mouse only plays a minor role mainly because of its limited size. The most common method to produce transgenic mammals is the microinjection of DNA constructs into the paternal pronucleus of a fertilized oocyte (Figure 1). The injected zygotes are implanted into the oviduct of foster mothers and brought to term. For mice and rats, about 20% of the offspring integrate the transgene into their genome and pass it to their offspring, thereby establishing a transgenic line. Several copies of the foreign DNA are integrated at one site in a chromosome. The expression of the transgene, however, does not only depend on the copy number but also on the chromosomal environment at the integration site and is therefore not absolutely predictable. In most cases the goal is to overexpress the gene of interest. The integration of the transgene with its own regulatory elements leads to an increased number of gene copies in the genetically modified animal and to a higher expression with conserved tissue specificity. To overexpress a gene in a specific organ or cell type, the respective cDNA is fused to a tissue-specific promoter and this construct is used for the production of transgenic animals. Table 1 summarizes examples of promoters used for transgene expression in different tissues. Furthermore, inducible promoter systems are available, which allow to control transgene expression by the application of substances to the genetically modified animals.

Recently, the microinjection technique has also been applied to downregulate gene expression in mice and rats. The method used is the expression of antisense RNAs in transgenic animals by the injection of con-

Table 1 - Promoters of tissue-specific expression of transgenes.

Promoter	Abbreviation	Cell type of highest activity	Reference
Neuron-specific enolase	NSE	Neurones	(112)
Tubulin α1	T-α1	Neurones	(113)
Glial-fibrillary acidic protein	GFAP	Astrocytes	(25,114,115)
Myosin light chain-2	MLC2	Cardiomyocytes	(116-118)
Preproendothelin-1	ET-1	Endothelial cells	(119)
Tie	tie	Endothelial cells	(120)
SM22α	SM22α	Vascular smooth muscle cells	(121)
α1-Antitrypsin	α1-AT	Hepatocytes	(91,122)
Albumin	ALB	Hepatocytes	(123)
Side-chain-cleavage enzyme	SCC	Steroidogenic cells	(124,125)
Kidney-androgen responsive protein	KAP	Renal proximal tubular cells	(126)

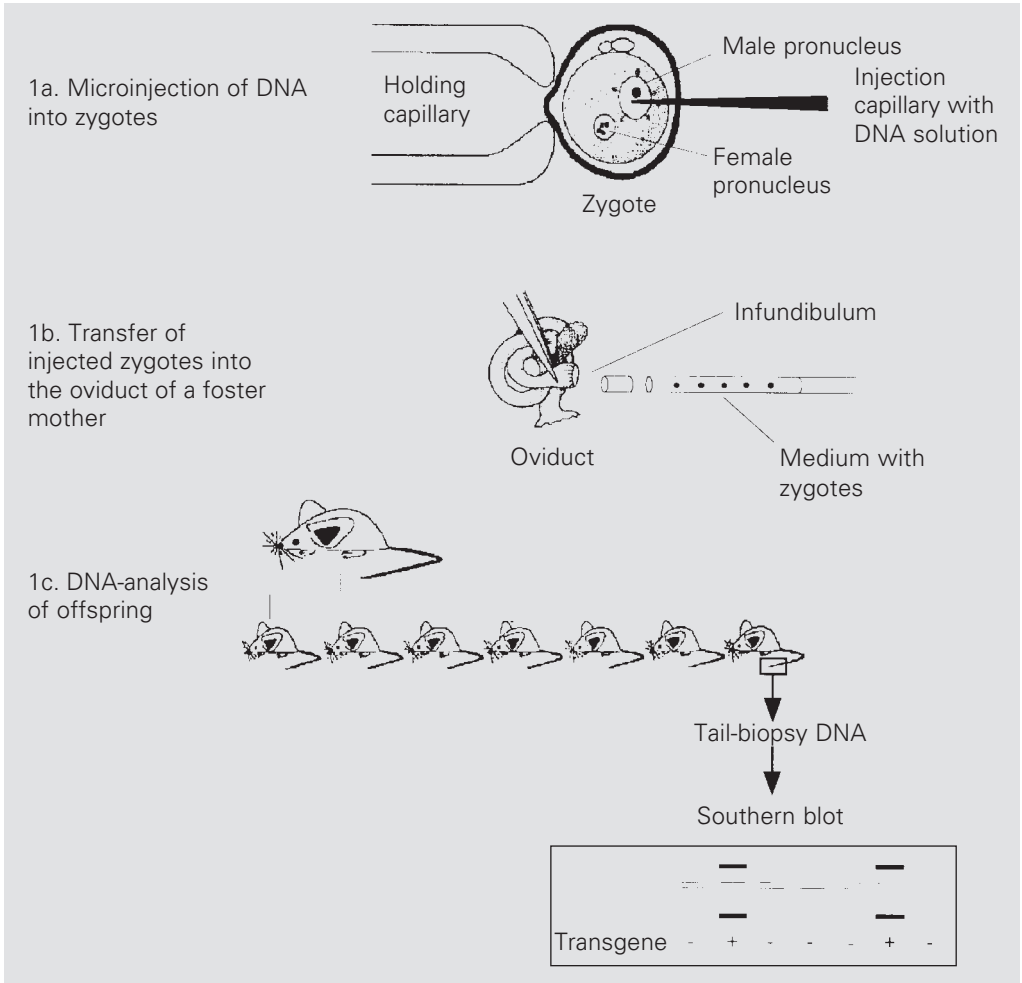

Figure 1 - Generation of transgenic rodents. A few copies of the DNA construct are injected into the male pronucleus (1a) of a fertilized oocyte, which is then transferred into the oviduct of a pseudopregnant foster mother (1b). The resulting offspring is analyzed for the presence of the transgene by Southern blotting with a specific probe (1c).

structs in which a part of the cDNA of interest has been fused in opposite orientation to a strong promoter. This results in the synthesis of an RNA complementary to the transcript from the gene of interest. The two RNAs will form a duplex in cells, which either blocks processing or translation of the mRNA or is a target for double-stranded RNA-editing enzymes (Figure 2). These enzymes covalently modify the mRNA in most cases leading to their functional destruction (21). Although the ablation of gene expression by antisense RNA is never complete, several animal models with physiological alterations have been successfully developed using this method also in the cardiovascular system (12,22-25) (see also below).

Gene targeting in embryonic stem cells (knockout technology)

The total ablation of genes was made possible by the recently developed knockout technology. Two new methods are the basis for this novel transgenic technique, the permanent culture of totipotent embryonic cells able to build up a whole animal (26,27) and the targeted disruption of a gene by homologous recombination (28,29). For homologous recombination a DNA construct containing large parts of the respective gene together with selectable marker genes is transfected into embryonic stem (ES) cells. In most cases the marker genes are used to create a null mutation of the targeted gene; however, also more subtle genetic alterations are possible. The cells, in which the very rare event of homologous recombination, i.e., the exchange of the endogenous gene with the DNA construct has taken place, are selected using the marker genes. The genotype is confirmed by PCR and Southern blotting and the mutated ES cells are microinjected into or fused with early embryos. After transfer to the uterus, the embryos are brought to term by foster mothers. The resulting chimeras between the host embryo and the ES cells are recognized by coat color and are used to develop a line of homozygous animals carrying the mutation in both alleles of the targeted gene after two subsequent generations. Such animals are the most powerful models to study the function of a gene. However, the method has also disadvantages: 1) the gene is mutated throughout ontogeny and in all organs of the animals. Thus, only the earliest role played by an essential gene during ontogeny can be detected in the knockout ani-

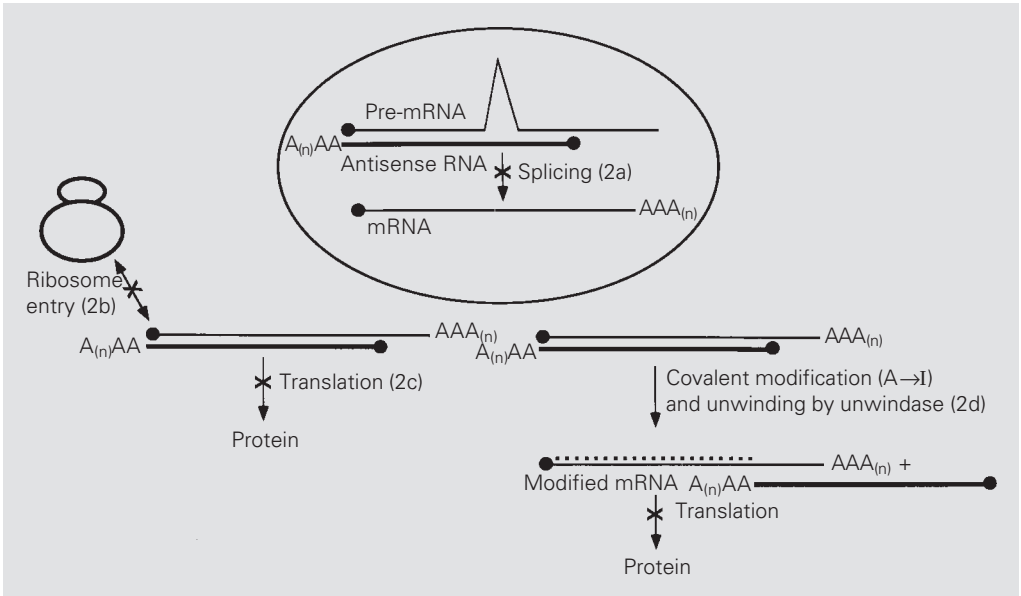

Figure 2 - Mechanisms of antisense RNA action. Antisense RNA covering two exons can inhibit splicing of the target pre-mRNA in the nucleus (2a). In the cytoplasm it can inhibit ribosome binding to the cap structure (closed circle) and the 5'-untranslated part of the mRNA (2b) or it can block the elongation of the nascent protein chain (2c). In addition, the extended double-stranded RNA formed by the antisense RNA and its target mRNA represent a substrate for double-stranded RNA editing enzymes (unwindase), which modify both RNAs by deaminating adenosine residues (21). The resulting inosine bases form base pairs with guanine leading to the synthesis of mutated proteins from such altered mRNAs (2d).

mals. Its function in adulthood cannot be studied since in many cases the homozygous animals will not reach this stage. To solve this problem conditional knockout techniques have very recently been designed and already successfully employed using the Cre-loxP system, a recombination system of bacteriophage P1, which allows the tissue-specific or developmental stage-dependent ablation of a gene (30). 2) Thus far, the technique is only available for the mouse and even in the mouse only cells derived from one strain (129/Sv) are routinely used for such experiments. A lot of effort has been devoted to the development of ES cells from other species, e.g. the rat (31), for the reasons already state above. However, germline-competent ES-cells could not be established from any other animal and there is no particular reason to explain this failure. The application of a novel technique on the basis of transgenic animals, which allows the selection of totipotent cells during the development of ES-cell lines may help solve this problem.

Transgenic and knockout animal models for the renin-angiotensin system

The generation of active angiotensin peptides is achieved by a small number of proteins all of which have been already overexpressed as well as inactivated by transgenic technology. The sole precursor angiotensinogen, synthesized mainly in the liver, is cleaved by renin produced by the juxtaglomerular cells of the kidney resulting in the liberation of the inactive decapeptide angiotensin I. The active peptide angiotensin II is generated by the proteolytic ablation of the two carboxyterminal amino acids of angiotensin I by the endothelium-associated angiotensin-converting enzyme (ACE). Angiotensin II elicits its effects via two different receptor subtypes, AT1, which is expressed in all main target organs like kidney, heart, brain, adrenal cortex, and vessel wall and represents the receptor responsible for nearly all known actions of the peptide, and AT2, which is mainly restricted to certain brain regions and the adrenal medulla. In the following section transgenic animals with changes in the expression of the genes for components of the renin-angiotensin system as well as the conclusions drawn from these models will be discussed.

Angiotensinogen

The angiotensinogen gene of mice (32), rats (33,34), and humans (11,35,36) has been expressed in transgenic mice (32,33,35,36) and rats (11) under the control of its own (11,32,34-36) or of the mouse metallothionein (mMT-1) promoter (33). All rodents transgenic for human angiotensinogen remained normotensive even though some of them exhibited very high levels of the human protein in plasma. This finding corroborated previous biochemical studies showing that human renin and angiotensinogen do not interact with their rodent counterparts (37, 38). Only when the animals carrying the human angiotensinogen gene are crossbred with human-renin transgenic rats or mice do the animals produce an excess of angiotensin II and become hypertensive (39-43; see below). The only transgenic animal carrying solely an angiotensinogen gene and being hypertensive was produced by Kimura et al. (34) by the introduction of the rat homolog into the mouse genome. Despite exhibiting equally high levels of circulating angiotensin II, the transgenic mouse model harboring the rat angiotensinogen gene under the control of the mMT-1 promoter is normotensive (33). The reason for this discrepancy may be the different tissue-specificity of expression of the two transgenes: while the foreign promoter leads to ectopic expression, the rat angiotensinogen promoter directs the production of additional angiotensin II to sites which are responsive to this

peptide. A tissue in which the correct site of expression is especially important is the brain. Using another transgenic animal model we could show that angiotensinogen in the brain plays an important role in blood pressure regulation. We produced a transgenic rat expressing an antisense RNA against angiotensinogen (44) exclusively in the brain with the help of the promoter for the glial-fibrillary associated protein (GFAP). High expression of this antisense RNA led to a brain-specific downregulation of angiotensinogen protein, a decrease in blood pressure (25), and a mild diabetes insipidus (45).

The decrease in blood pressure in these rats was comparable to the decrease observed in mice carrying only one functional angiotensinogen allele (46). They were produced in a gene-titration experiment together with mice having zero, two, three, and four alleles of this gene. Circulating angiotensinogen levels as well as blood pressure correlated strongly with the gene dose, supporting the important role of this protein in blood pressure regulation at least in rodents. Very low blood pressure levels were measured in angiotensinogen-knockout mice which were produced by three groups independently (46-48). Depending on the genetic background the targeted ablation of this protein even produced a lethal phenotype, the cause of which is not yet clarified but may be related to morphological alterations observed in the kidney. Several groups have rescued the phenotype of these knockout mice by cross-breeding them with transgenic mice expressing the rat angiotensinogen gene (49) or the human renin and angiotensinogen genes (50). The latter experiment resulted in mice in which the only active renin-angiotensin system is derived from human genes.

Renin

Transgenic mice (33,40,43,51-57) and rats (9,11) carrying renin genes from rats (33), mice (9,54-57), and man (11,40,43,51-53) have been generated. The first animal models were designed to analyze the pattern of differential expression of mouse renin genes. The mouse is the only species analyzed so far with more than one renin gene. There are strains with one renin gene, *Ren*-1^c, and strains bearing two genes, *Ren*-1^d and *Ren*-2, closely linked on chromosome 1 since they originate from an evolutionarily recent gene duplication. The genes are differentially expressed and transgenic experiments showed that DNA sequences in the promoter as well as in the transcribed regions are required for a correct tissue specificity of expression. Transgenic mice with the whole *Ren*-2 gene including 5.3 (54) or 2.5 kb (55,56) of the promoter region and all exons and introns or with a 4.6-kb promoter fragment and the SV40 T-antigen as a reporter gene express the transgene correctly (58-61), while animals with only 2.5 kb of the promoter and the same reporter gene show ectopic expression (62). Thus, there seems to be a redundancy of tissue-specific elements in the promoter and in the transcribed region as 2.5 kb of promoter are only sufficient for correct expression in concert with all exons and introns, while longer 5'-flanking regions are independent of other parts of the *Ren*-2 gene. Recent studies have revealed possible candidates for such tissue-specific elements in the distant promoter (63) as well as in intron I of the gene (64-66). Comparable results have also been published for the *Ren*-1^d gene as 5 kb of the promoter led to the correct expression in transgenic mice only in the presence of the whole transcribed region but not fused to a chloramphenicol acetyltransferase (CAT) reporter gene (57).

While for none of these mouse models blood pressure values have been reported, the arterial pressure of mice carrying the rat renin gene under the control of the mMT-1 promoter was determined and found to be normal, which may be explained by species specificity of the renin-angiotensinogen interaction (33). When these mice were crossed

with mMT-1-angiotensinogen transgenic animals (see above) the double transgenic mice became hypertensive. The same holds true for double transgenic mice and rats with the human renin and angiotensinogen transgenes also because of the species specificity of the enzyme-substrate reaction (39,40, 42,43). These "humanized" rodent models independently developed by several groups are useful tools to study the local production and action of angiotensin II in tissues and to test human renin inhibitors, which cannot be tested in normal rodents because of species specificity (11,67). In addition, such animals may help elucidate the cause for specific forms of pregnancy-induced hypertension (68).

Another important model for the study of tissue-based renin-angiotensin systems is a transgenic rat carrying the murine *Ren*-2 gene, TGR(mREN2)27 (9). These animals develop severe hypertension and cardiovascular hypertrophy despite low levels of circulating angiotensin II. However, the generation of this peptide is massively enhanced in several tissues, like adrenal glands and brain, leading to numerous physiological changes (69-90). These changes as well as the very high circulating levels of prorenin, the inactive precursor of renin, may contribute to the hypertensive phenotype of TGR(mREN2)27, although its etiology has not been fully clarified. Veniant et al. (91) have shown that prorenin when expressed in the liver of transgenic rats with the use of the α1-antitrypsin promoter and reaching similarly high circulating levels can cause a degree of cardiovascular hypertrophy comparable to that observed in TGR(mREN2)27 probably because it can be activated in peripheral tissues. Others have presented evidence that elevated angiotensin levels in brain (73,77, 78), kidney (69,76,77), and adrenal gland (74,75,77,89) of TGR(mREN2)27 also play important roles in the development of hypertension. Studies using the specific downregulation of the renin-angiotensin system in single organs like the one presented above (25) may help elucidate the relative importance of the local angiotensin-generating systems in the pathogenesis of hypertension in TGR(mREN2)27.

As mentioned above, ES cells are almost exclusively from strain 129/Sv, which contains two renin genes. Although both genes have been separately inactivated by gene targeting, a total renin-knockout by simple crossbreeding is precluded by the close linkage of the two genes. The *Ren*-2 knockout mice are healthy and normotensive and only exhibit increased active renin and reduced prorenin levels in plasma (92). Animals lacking *Ren*-1^{d} have been produced by two groups independently (93,94). While the resulting animals were normotensive in one experiment (93), in the other study, they showed morphological alterations in the kidney exemplified by a lack of secretory granules in the juxtaglomerular cells and hypertrophy of the macula densa as well as enhanced circulating prorenin levels, and the females became slightly hypotensive (94). The quite mild phenotype of both knockout models argues in favor of a high redundancy of the two renin genes, i.e., the presence of one gene can largely compensate for the lack of the other.

ACE

Transgenic rats overexpressing ACE predominantly in the heart have recently been produced and the phenotype has been partially reported (95). Despite very high levels of ACE activity in the heart there are no morphological alterations of this organ unless it is pressure overloaded by aortic banding. This treatment results in a significantly higher hypertrophic response in ACE-transgenic rats than in control animals, supporting the important role of angiotensin II in this process postulated by earlier pharmacological and transgenic (see below) studies.

Transgenic mice were generated to analyze the testis-specific promoter in intron 13 of

the ACE gene responsible for the production of a shorter but still active protein in this organ (96-99). ACE seems to play an important role in the testis since male ACE-knockout mice are infertile (100). Furthermore, the animals are hypotensive and develop kidney abnormalities comparable to those of the angiotensinogen knockout animals (101). In a very recent experiment the same phenotype could be shown for mice in which only the membrane anchor of the ACE-protein was deleted by knockout technology (102). These animals have still normal circulating ACE levels but lack the membrane-bound form, which, according to this report, is the only functionally important isoform of the enzyme.

Angiotensin receptors

The only reported transgenic animal models overexpressing AT1-receptors have targeted its expression to the heart by the use of the α-myosin-heavy chain promoter (103, 104). However, the phenotypes of the transgenic mouse and the transgenic rat thus generated were dramatically different. The mice exhibit a drastic cardiac hypertrophy and die of severe bradycardia early in life (103), whereas the rats appear absolutely normal unless the heart is pressure overloaded by aortic banding which, as is the case for ACE-transgenic rats, leads to a more pronounced hypertrophy than in control animals (104). The difference might be related to the different source of the AT1 receptor: while for the transgenic mouse the murine AT1A-cDNA receptor was used, the rat overexpressed the human homolog.

When the AT2 receptor is overexpressed in the heart the resulting transgenic mice show no obvious morphological alterations but they are less sensitive to angiotensin-II induced blood-pressure elevation indicating that the AT2 receptor counteracts the AT1 receptor at least in this respect (105).

Several groups have inactivated the AT1 receptor by gene targeting in mice (106-108). Like for the renin gene, these studies are hampered by the existence of two different genes coding for AT receptors in this species and also in rats, AT1A and AT1B. The knockout experiments revealed, however, that the AT1A receptor is the more important isoform since the gene-targeted mice showed a comparably severe phenotype as angiotensinogen- or ACE-knockout animals. Furthermore, mice lacking AT1B show no obvious phenotype (109). Double knockout animals will elucidate whether the slightly less severe kidney alterations observed in AT1A-deficient mice compared to mice lacking angiotensinogen are due to a compensatory action of AT1B receptors in this organ.

The AT2 receptor has also been inactivated by two groups independently (110, 111). In addition to behavioral abnormalities, AT2 deficiency results in a more pronounced pressor response to angiotensin-II infusion, corroborating the antagonism between AT1 and AT2.

Conclusions

The transgenic animal models for the renin-angiotensin system established so far have provided novel information concerning the function of this peptide system active in cardiovascular regulation. The most important finding is the importance of tissue-based angiotensin generation in contrast to the circulatory system. Tissue-specific ablation of the renin-angiotensin system by antisense RNA expression or conditional knockout technology will facilitate the study of these functionally important peptide systems, e.g., in the brain, adrenal gland, vascular wall, heart, and kidney. Existing as well as future animal models will increase our understanding of the basic cardiovascular regulation and of the mechanisms involved in the development of hypertension and may be of help to design new therapeutic strategies for the therapy of cardiovascular diseases.

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