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Dimethylarginine metabolism during acute and chronic rejection of rat renal allografts

Dariusz Zakrzewicz[1,*], Anna Zakrzewicz[2,*], Sigrid Wilker[2], Rolf-Hasso Boedeker[3], Winfried Padberg[2], Oliver Eickelberg[4] and Veronika Grau[2]

[1]Department of Medicine II, University of Giessen Lung Center, Aulweg 123, D-35392 Giessen, Germany, [2]Laboratory of Experimental Surgery, Department of General and Thoracic Surgery, Justus-Liebig-University Giessen, Rudolf-Buchheim-Str. 7, D-35385 Giessen, Germany, [3]Institute for Medical Informatics, Section of Medical Statistics, Justus-Liebig-University Giessen, Heinrich-Buff Ring 44, D-35392 Giessen, Germany and [4]Comprehensive Pneumology Center, Ludwig-Maximilians-University, Asklepios Hospital, and Helmholtz Zentrum München, Ingolstädter Landstr 1, D-85764 Neuherberg/München, Germany

Correspondence and offprint requests to: Veronika Grau; E-mail: veronika.grau@chiru.med.uni-giessen.de
[*]These authors contributed equally to this study.

Abstract

Background. Dimethylarginines are inhibitors of NO synthesis and are involved in the pathogenesis of vascular diseases. In this study, we ask the question if asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels change during fatal and reversible acute rejection, and contribute to the pathogenesis of chronic vasculopathy.
Methods. The Dark Agouti to Lewis rat strain combination was used to investigate fatal acute rejection. Fischer 344 kidneys were transplanted to Lewis rats to study reversible acute rejection episode and the process of chronic rejection. Isograft recipients and untreated Lewis rats were used as controls. L-arginine derivatives were determined by HPLC, and ADMA-metabolizing enzymes were studied by quantitative RT–PCR and western blotting.
Results. Renal transplantation transiently increased dimethylarginine levels independent of acute rejection. ADMA plasma levels did not importantly differ between recipients undergoing fatal or reversible acute rejection, whereas SDMA was even lower in recipients of Fisher 344 grafts. In comparison to isograft recipients, ADMA and SDMA levels were slightly elevated during reversible, but not during the process of chronic rejection. Increased dimethylarginine levels, however, did not block NO synthesis. Interestingly, protein methylation, but not ADMA degradation, was increased in allografts.
Conclusions. Our data do not support the concept that renal allografts are protected from fatal rejection by dimethylarginines. Dimethylarginines may play a role in triggering chronic rejection, but a contribution to vascular remodelling itself is improbable. In contrast, differential arginine methylation of yet unknown proteins by PRMT1 may be involved in the pathogenesis of acute and chronic rejection.

Keywords: ADMA; kidney transplantation; L-arginine; rat; SDMA

Introduction

Dimethylarginines have moved into the spotlight of scientific interest as endogenous inhibitors of nitric oxide synthesis. They are potential mediators of endothelial dysfunction, hypertension and vascular remodelling, and seem to be involved in chronic kidney diseases [1–7]. NO synthesis involves cellular uptake of L-arginine (L-arg) by y^+ transporters, which is inhibited by asymmetric dimethylarginine (ADMA) and by symmetric dimethylarginine (SDMA) [1,6]. These transporters are also needed for renal L-arg absorption and contribute to the maintenance of systemic L-arg levels. L-arg can be converted to NO and citrulline by NOS [2].

Dimethylarginine metabolism is also of outstanding interest in the context of transplantation, predominantly because the NOS isoforms, endothelial NOS (eNOS) and

inducible NOS (iNOS), play protective and deleterious roles in acute and chronic allograft rejection [8–14]. Acute and chronic allograft rejection are linked to each other, as acute rejection is an important clinical risk factor for the development of chronic rejection [15] and experimental evidence suggests that chronic rejection is irreversibly triggered during acute rejection episodes [16]. An important hallmark of chronic rejection of kidneys is the development of allograft vasculopathy, a severe intimal hyperplasia of renal arteries. The idea suggests itself that increased levels of dimethylarginines may play a role in the pathogenesis of these lesions. Indeed, clinical and experimental data evidenced that dimethylarginines contribute to cardiac allograft vasculopathy [17,18], which resembles vasculopathy of renal allografts [19].

ADMA and SDMA are elevated in patients suffering from renal disease [3,20–24]. Moreover, ADMA levels correlate negatively with renal function, and positively with mortality and cardiovascular complications [25]. In patients undergoing dialysis, ADMA and SDMA are also increased [23]. Transplantation reduces SDMA [26], whereas its effect on ADMA is disputed [23,24,26,27].

ADMA synthesis starts with the methylation of protein arginine residues by protein arginine methyltransferases (PRMTs) [28], which catalyse the formation of monomethylarginine (MMA) from L-arg [29]. These enzymes are classified into type I (PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8) and type II (PRMT5, PRMT7 and FBXO10). Type I PRMTs produce ADMA, while type II PRMTs produce SDMA [28]. After proteolytic degradation of methylated proteins, MMA, SDMA or ADMA are released and in part cleared by renal excretion [30]. In addition, ADMA, but not SDMA, is degraded in the liver, kidney, and other organs to citrulline and dimethylamines by dimethylarginine dimethylaminohydrolases (DDAH), DDAH1, and DDAH2 [31,32].

The purpose of this study is to answer two questions: (i) do dimethylarginine levels change during fatal and reversible acute allograft rejection, and (ii) do dimethylarginines and their metabolism contribute to the pathogenesis of chronic vasculopathy? Therefore, we investigate dimethylarginine metabolism during renal allograft rejection as well as NOS expression and activity. An experimental model of fatal acute rejection of rat renal allografts is compared with a model for chronic allograft rejection, which involves a reversible rejection episode: (i) kidney transplantation in the fully allogenic, Dark Agouti (DA) to Lewis (LEW) rat strain combination leads to acute graft destruction 4–5 days post-transplantation. Graft recipients are investigated on Day 4. (ii) Kidneys transplanted in the Fischer 344 (F344) to LEW combination remain functional for at least 6 months but undergo reversible acute rejection around Day 9 [33], and develop allograft vasculopathy in the long run [34]. Those recipients are investigated on Day 9 and during vascular remodelling on Day 42.

Materials and methods

Details of materials and methods used in these studies are given in the online supplementary data.

Animal experiments

LEW (RT1[1]), DA (RT1[av1]) and F344 (RT1[1v1]) male rats weighing 260–300 g from Harlan Winkelmann (Borchen, Germany) were kept under conventional conditions. Animals received humane care following the current version of the German Law on the Protection of Animals and the 'Principles of Laboratory Animal Care' by the National Society for Medical Research as well as the NIH 'Guide for the Care and Use of Laboratory Animals'.

Kidneys were transplanted orthotopically to totally nephrectomized LEW recipients as described [35,36]. DA or F344 rats were used as donors for allogenic transplantation, and LEW rats for isogenic transplantation. Total ischaemic times remained below 30 min. Recipients of DA kidneys died 7.4 ± 0.7 days (mean $\pm$ SD, $n = 10$) after surgery, whereas F344 grafts remained functional for at least 180 days. Untreated healthy LEW rats served as controls.

On Day 4, 9 or 42 after transplantation, animals were anaesthetized with 60 mg/kg sodium pentobarbital i.p. (Narcoren, Merial, Hallbergmoos, Germany) and obtained 1000 IU/kg heparin i.v. (Ratiopharm, Ulm, Germany). Blood was taken by heart puncture and centrifuged at $4750\,g$ for 10 min, and plasma was stored at $-20°C$. To assess renal function, plasma creatinine and urea were measured. A detailed description of the method is given in the online supplementary data. Pieces of kidneys were snap-frozen and stored at $-80°C$.

Immunohistochemistry

Immunohistochemical detection of PRMT1, and identification of monocytes and T cells were essentially performed as described [33,37].

Quantification of ADMA, SDMA and L-arg

Isolation of basic amino acids and derivatization were performed as described [32,38]. ADMA, SDMA and L-arg plasma levels were determined by high-performance liquid chromatography (HPLC).

DDAH activity

DDAH activity in tissue extracts was measured by an HPLC-based method [32].

Quantitative RT–PCR

Details on the reverse transcriptase reaction, quantitative RT–PCR and primer sequences are available in the online supplementary data.

Immunoblotting

Protein extraction and immunoblotting were performed essentially as described [32].

Quantification of nitrite/nitrate (NO_x)

To estimate NO production, NO_x was determined by the Griess reaction [39].

Statistics

Statistical evaluation was performed to answer the following questions: (i) do acute or chronic rejection change the location of the distribution of the parameters of interest? Data from isograft recipients are compared with allograft recipients at the same time after transplantation. (ii) Does the location of the parameters differ among animals undergoing fatal acute (Day 4, DA to LEW) and reversible acute rejection (Day 9 allografts, F344 to LEW)? (iii) Does the location of the parameters change between Day 9 and Day 42 after allogenic transplantation? (iv) Do isogenic transplantation and recipient nephrectomy change the location of the distribution of the parameters of interest in comparison to untreated controls? For this purpose, data from control animals were compared with isograft recipients (Day 4, 9 and 42). The distribution of the observed data was described by median, minimum and maximum. The hypothesis that there is no difference between the groups of interest was tested using the non-
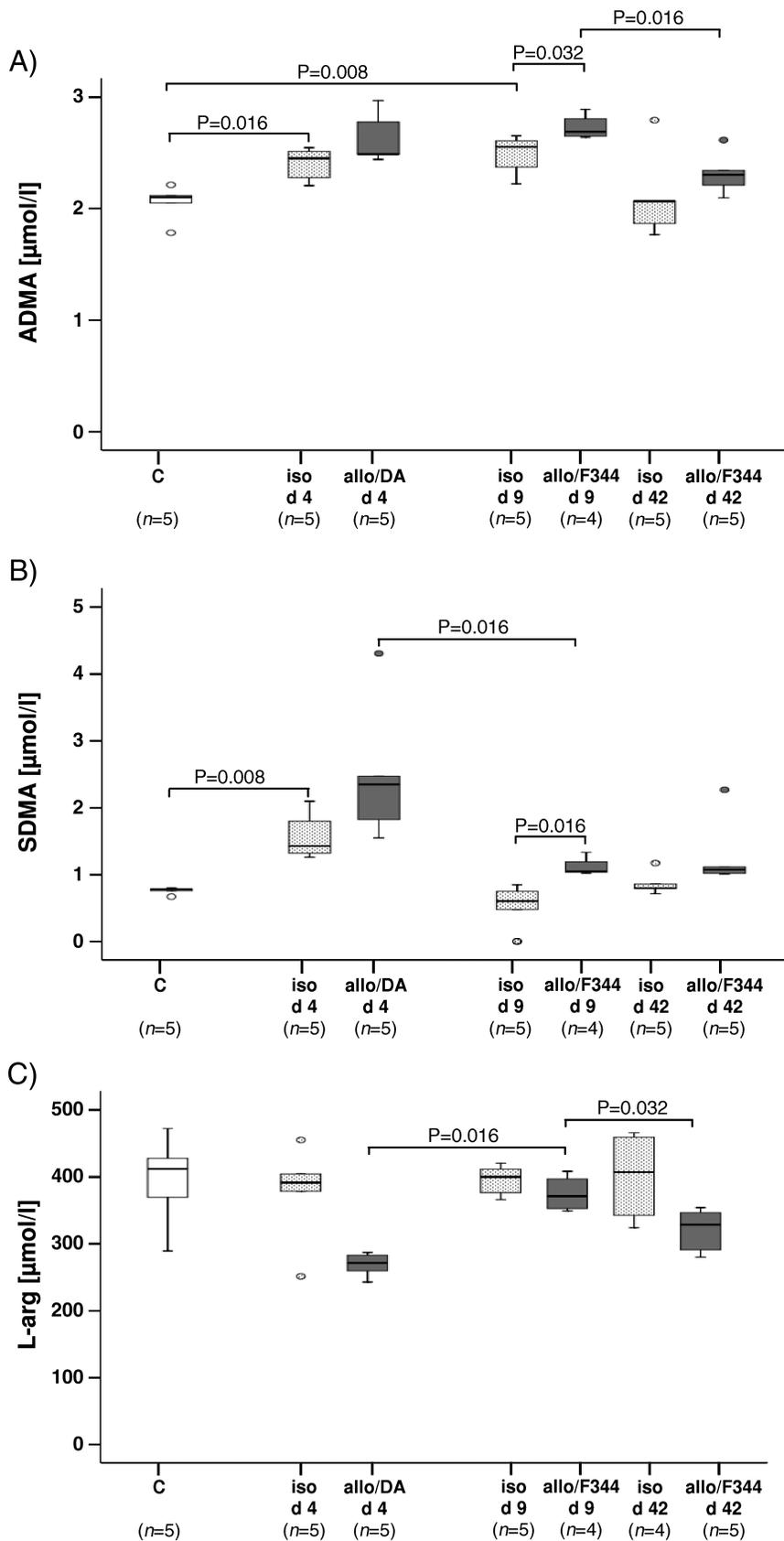
Fig. 1. ADMA, SDMA and L-arg plasma concentration. ADMA (**A**), SDMA (**B**) and L-arg (**C**) were quantified by HPLC in the blood plasma from healthy control rats (C, white), isograft recipients (iso, dotted), and allograft recipients of DA and F344 donors (allo, grey). Graft recipients were investigated on Day (d) 4, 9 and 42 post-transplantation. Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); circles indicate data beyond 3 × standard deviation; P-values ≤0.05 and number of animals (*n*) used in the study are indicated.

Table 1. L-arg/ADMA, L-arg/SDMA, and ADMA/SDMA ratios in the plasma of control rats and renal graft recipients

	Median (min–max) (P-value)		
	L-arg/ADMA	L-arg/SDMA	ADMA/SDMA
Control	201 (137–229) (n = 5)	548 (360–602) (n = 5)	2.6 (2.6–2.9) (n = 5)
Iso, Day 4	164 (110–178) (0.095)[a] (n = 5)	265 (139–345) (0.008)[a] (n = 5)	1.5 (1.1–2.0) (0.008)[a] (n = 5)
Allo DA, Day 4	97 (93–113) (0.032)[b] (n = 5)	104 (66–175) (0.032)[b] (n = 5)	1.1 (0.6–1.5) (0.151)[b] (n = 5)
Iso, Day 9	156 (144–177) (0.151)[a] (n = 5)	592 (485–768) (0.286)[a] (n = 5)	3.7 (3.1–4.6) (0.016)[a] (n = 5)
Allo F344, Day 9	138 (123–149) (0.063)[b] (0.016)[c] (n = 4)	354 (267–389) (0.029)[b] (0.016)[c] (n = 4)	2.5 (2.1–2.6) (0.029)[b] (0.016)[c] (n = 4)
Iso, Day 42	174 (161–263) (0.730)[a] (n = 4)	432 (385–653) (0.730)[a] (n = 4)	2.4 (2.3–2.6) (0.008)[a] (n = 5)
Allo F344, Day 42	138 (121–159) (0.016)[b] (1.000)[d] (n = 5)	322 (123–351) (0.016)[b] (0.190)[d] (n = 5)	2.1 (1.0–2.5) (0.095)[b] (0.190)[d] (n = 5)

The number of animals (n) used in the study is indicated.
[a]Isograft recipient versus control.
[b]Allograft versus isograft.
[c]Allograft Day 4 versus allograft Day 9.
[d]Allograft Day 9 versus allograft Day 42.

Table 2. Urea and creatinine concentration in the plasma of control rats and renal graft recipients

	Median (min–max) (P-value), mg/dL	
	Urea	Creatinine
Control	36 (30–41) (n = 5)	0.50 (0.40–0.50) (n = 5)
Iso, Day 4	49 (42–101) (0.008)[a] (n = 5)	0.70 (0.60–1.00) (0.008)[a] (n = 5)
Allo DA, Day 4	58 (53–565) (0.310)[b] (n = 5)	0.90 (0.80–4.90) (0.151)[b] (n = 5)
Iso, Day 9	57 (48–64) (0.008)[a] (n = 5)	0.60 (0.50–0.70) (0.016)[a] (n = 5)
Allo F344, Day 9	98 (77–108) (0.016)[b] (0.286)[c] (n = 4)	0.85 (0.80–1.00) (0.016)[b] (0.556)[c] (n = 4)
Iso, Day 42	39 (35–49) (0.151)[a] (n = 5)	0.60 (0.60–0.70) (0.008)[a] (n = 5)
Allo F344, Day 42	66 (59–178) (0.008)[b] (0.286)[d] (n = 5)	0.80 (0.70–1.40) (0.032)[b] (0.413)[d] (n = 5)

The number of animals (n) used in the study is indicated.
[a]Isograft recipient versus control.
[b]Allograft versus isograft.
[c]Allograft Day 4 versus allograft Day 9.
[d]Allograft Day 9 versus allograft Day 42.

parametric exact Mann–Whitney U-test (SPSS software version 15). Data were analysed in exploratory manner.

Results

Histopathology

Day 4 renal allografts (DA to LEW) were characterized by severe perivascular and interstitial mononuclear infiltrates, and isografts were almost unimpaired [36]. After transplantation in the F344-to-LEW rat strain combination, which results in chronic rejection in the long run, severe but reversible acute rejection developed peaking around Day 9 after surgery [33]. Day 9 histopathology resembled Day 4 DA-to-LEW kidneys, and the infiltrate was predominantly composed of macrophages and T cells (Supplementary Figures 1–3). On Day 42 (F344 to LEW), the infiltrate was reduced, and macrophages and T cells still surrounded blood vessels and exhibited a patchy distribution in the renal interstitium (Supplementary Figures 1–3). Day 9 and Day 42 isografts were almost normal (Supplementary Figures 1–3; see online supplementary material for a colour version of these figures).

ADMA, SDMA and L-arg plasma levels

In healthy control rats, the ADMA concentration was 2.10 (1.78–2.21)µmol/L, similar to previously published observations [40,41]. In comparison to controls, ADMA levels were increased in isograft recipients on Day 4 and 9 after transplantation, but returned to control levels on Day 42 (Figure 1A). Plasma ADMA levels of Day 9 allograft (F344) recipients were higher compared with Day 9 isograft and Day 42 allograft recipients (Figure 1A).

On Day 4, plasma SDMA levels were increased in isograft recipients compared with controls (Figure 1B). However, our data did not suggest that Day 4 allograft recipients differ from isograft recipients. SDMA levels attained control levels in Day 9 and 42 isograft recipients, but were elevated in Day 9 F344 allograft recipients compared with isograft recipients.

L-arg plasma levels tended to be lower 4 days after allogenic transplantation compared with isograft recipients (P = 0.095) and with F344 allograft recipients on Day 9 (Figure 1C). L-arg/ADMA and the L-arg/SDMA ratios are indicated in Table 1. L-arg/ADMA ratios as well as L-arg/SDMA ratios were decreased in plasma samples from allograft recipients during fatal acute rejection compared

A)

B)

C)

D)
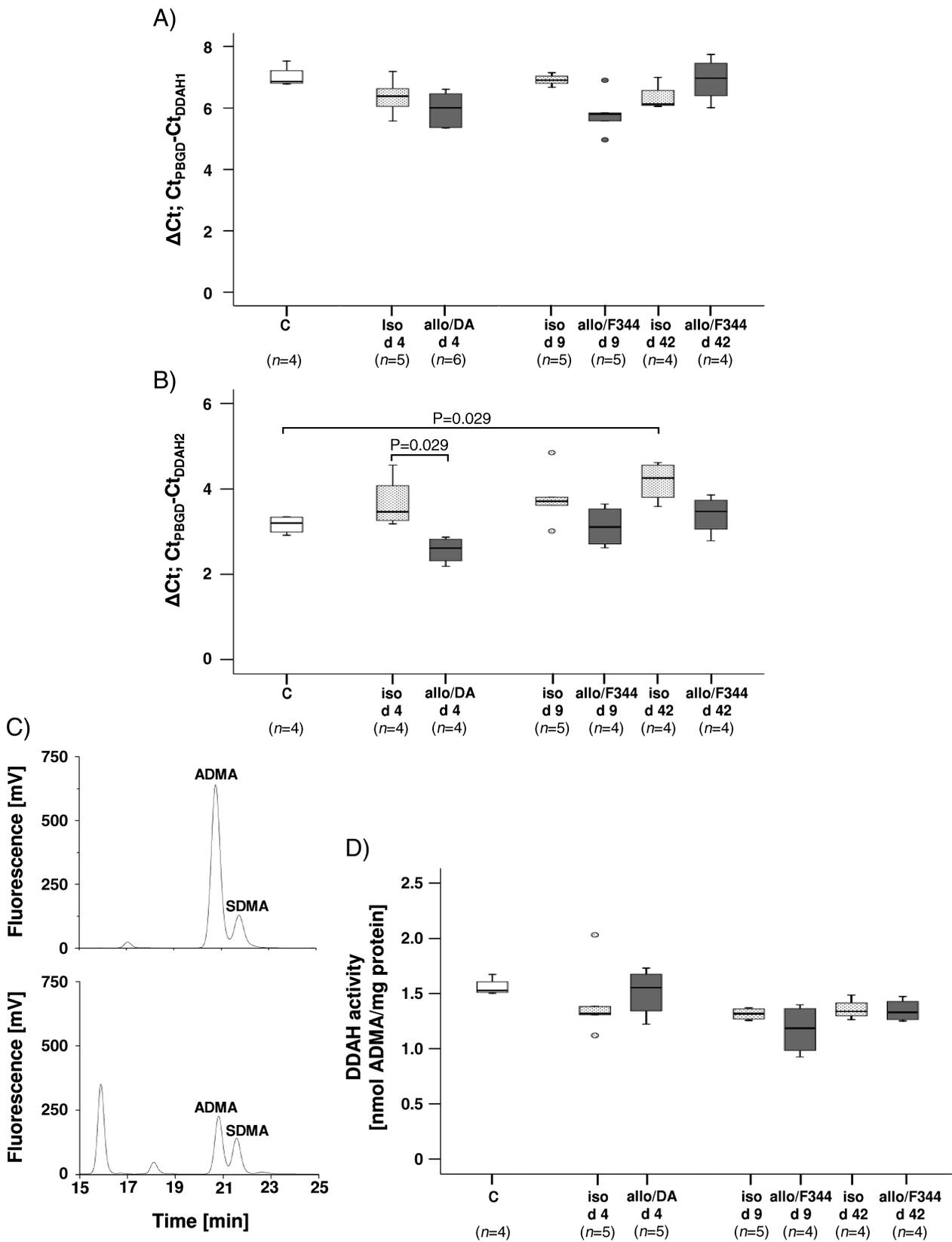
with the respective isograft recipients and with recipients of F344 allografts on Day 9 (Table 1).

Renal function

Plasma creatinine and urea concentrations were increased in all isograft recipients, except in Day 42 allografts, compared with untreated controls (Table 2). In comparison to isograft recipients, both urea and creatinine levels were increased in recipients of F344 kidneys on Day 9 and 42 (Table 2).

DDAH mRNA expression and activity

Quantitative RT–PCR did not confirm the assumption that DDAH1 mRNA expression in renal tissue differs between experimental groups (Figure 2A). In contrast, DDAH2 mRNA levels were lower in Day 4 allografts compared with isografts (Figure 2B). Renal DDAH function was analysed in vitro (Figure 2C and D), and equally high rates of ADMA degradation were observed in all experimental groups (Figure 2C and D). As expected, SDMA was not degraded (Figure 2C, lower panel).

PRMT1 protein expression

PRMT1 protein levels were examined by western blotting (Figure 3A, C and E). In both models, kidney allografts were characterized by an increase in PRMT1 expression, compared with appropriate isografts (Figure 3B, D and F). Of note, also Day 42 F344 allografts expressed higher PRMT1 levels (Figure 3F).

Immunohistochemistry using the same antibodies to PRMT1 was performed on renal isografts and allografts (Figure 4, Supplementary Figures 2 and 3). PRMT1 immunoreactivity was ubiquitously seen in all renal grafts. Besides a moderate cytoplasmic staining, a more conspicuous nuclear signal was present in numerous but not all cells. Structures morphologically compatible with distal tubules and collecting ducts as well as single glomerular cells were strongly immunopositive. Additionally, intensely stained cells were detected in perivascular regions and in the renal interstitium of all allografts. Numerous macrophages and T lymphocytes were detected in the same regions, suggesting that these strongly PRMT1-positive cells belong to the infiltrate (Figure 4, Supplementary Figures 2 and 3). PRMT1-positive infiltrates were less abundant in Day 42 F344 allografts (Supplementary Figures 2 and 3).

Protein methylation in renal tissue

To estimate renal PRMT function, methylated proteins were analysed by western blotting using antibodies to dimethylarginine–glycine repeats [42] (Supplementary Figure 4). Protein methylation increased during acute rejection. Most strikingly, in Day 42 allografts undergoing chronic rejection, several proteins exhibited impaired methylation. In addition, differences in the methylation of individual proteins were observed between control kidneys, isografts and allografts (details are given in Supplementary Tables 1–3).

Expression of iNOS and eNOS

Protein levels of iNOS and eNOS were examined by western blotting. As expected, iNOS was induced in DA allografts during fatal acute rejection (Figure 5A). No iNOS was detected in Day 9 and 42 F344 grafts (Figure 5B and C). In contrast, eNOS was equally expressed by Day 4 isografts and allografts (Figure 5D and E). A slight increase in eNOS expression was detected in Day 9 isografts, which in turn did not differ from Day 42 isografts (Figure 5F–I).

Tissue and plasma NO_x

Our experiments did not detect differences in NO_x plasma concentrations between controls and all isograft recipients (Figure 6A). During acute rejection, however, NO_x levels markedly increased in allograft recipients of both models compared with isograft recipients (Figure 6A). As expected, NO_x levels were lower in recipients of F344 grafts compared with recipients of DA grafts. Our data suggest that NO_x returns to isograft recipient levels in F344 allograft recipients on Day 42.

Also, NO_x isograft tissue levels did not differ from controls (Figure 6B). NO_x levels increased in allografts undergoing fatal acute rejection (Figure 6B), but in Day 9 and 42 F344 kidneys, NO_x levels were not elevated compared with isografts (Figure 6B).

Discussion

The most important findings of this study are (i) shortly after renal transplantation and recipient nephrectomy, ADMA and SDMA are increased, even in the absence of acute rejection. (ii) ADMA levels did not importantly differ between recipients undergoing fatal and reversible acute rejection, whereas SDMA concentrations are lower during reversible rejection. (iii) During reversible acute rejection preceding chronic allograft rejection, ADMA and SDMA plasma levels are slightly elevated in comparison with isograft recipient plasma. (iv) PRMT1 protein expression and function are increased in DA and in F344 allografts compared with isografts, probably due to graft-infiltrating leucocytes. (v) In spite of elevated systemic dimethylarginine concentrations, NOS is at least partially functional.

Fig. 2. DDAH mRNA expression and activity in renal grafts. DDAH1 (**A**) and DDAH2 (**B**) mRNA expression were analysed by qRT–PCR. Data are expressed as the ΔCt of PBGD as a house-keeping gene, and DDAH1 or DDAH2. Healthy control kidneys (C, white), isografts (iso, dotted), and allografts of DA and F344 donors (allo, grey) were investigated on Day (d) 4, 9 and 42 post-transplantation. (**C, D**) For the measurement of DDAH activity, excess amounts of ADMA and SDMA were added to protein extracts form control kidneys, isografts and allografts, and incubated for 2 h at 37°C. Thereafter, ADMA and SDMA were measured by HPLC. (C) HPLC chromatograms of a typical experiment: the upper panel shows a sample before, and the lower panel after incubation at 37°C. Note that in contrast to ADMA, SDMA is not degraded. (D) Quantification of ADMA degradation; circles indicate data beyond 3 × standard deviation; P-values ≤0.05 and number of animals (n) used in the study are indicated. (A, B, D) Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers).
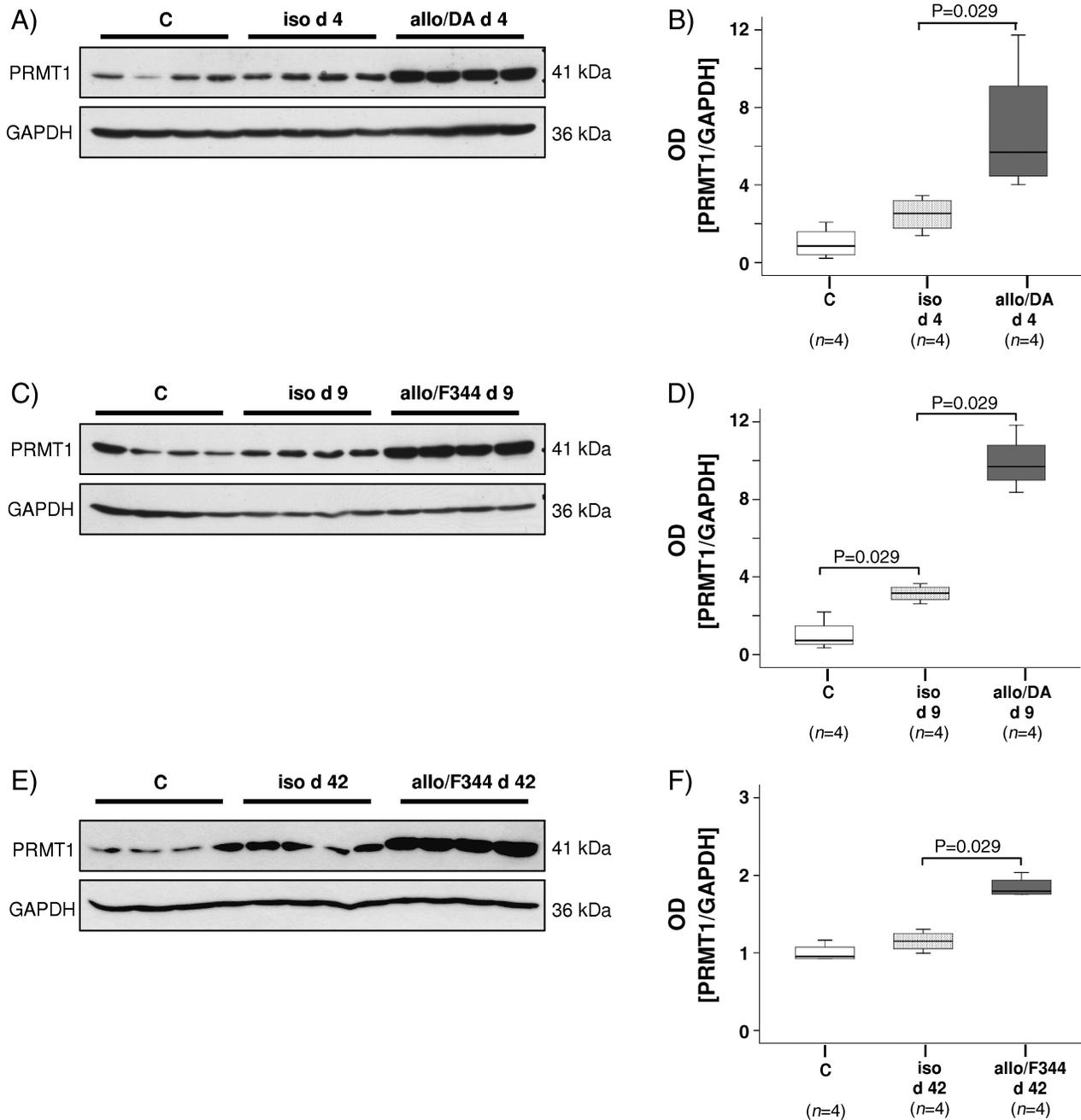
Fig. 3. PRMT1 expression in renal tissue. Homogenates of healthy control kidneys (C, white), isografts (iso, dotted), and allografts of DA and F344 donors (allo, grey) were separated by SDS–polyacrylamide (12%) gel electrophoresis. PRMT1 expression was (**A**, **C**, **E**) analysed by western blotting on Day (d) 4, 9 and 42 post-transplantation. The intensity of the resulting bands (**B**, **D**, **F**) was quantified by densitometry and divided by the values obtained for the house-keeping protein GAPDH. The mean of the PRMT1/GAPDH ratio of the controls was set to 1, and each individual value including the control values was calculated accordingly. Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); P-values ≤ 0.05 and number of animals (*n*) used in the study are indicated.

Concerning the question whether changes in dimethylarginine levels and their metabolism are involved in the pathogenesis of acute or chronic kidney rejection, only differences between isograft and allograft recipients are relevant (Supplementary Figure 5). The most striking differences are seen for PRMT1 expression and protein arginine methylation, which strongly increase in allograft recipients during fatal and reversible acute rejection and slightly increase during the process of vascular remodelling on Day 42. The resulting increased protein methylation, however, does not consistently lead to elevated dimethylarginine levels, which are only seen during reversible acute rejection. Accordingly, increased dimethylarginine levels may be involved both in reverting acute rejection and in triggering chronic rejection in F344 to LEW allografts at Day 9. As the changes in dimethylarginines are very small, it can,
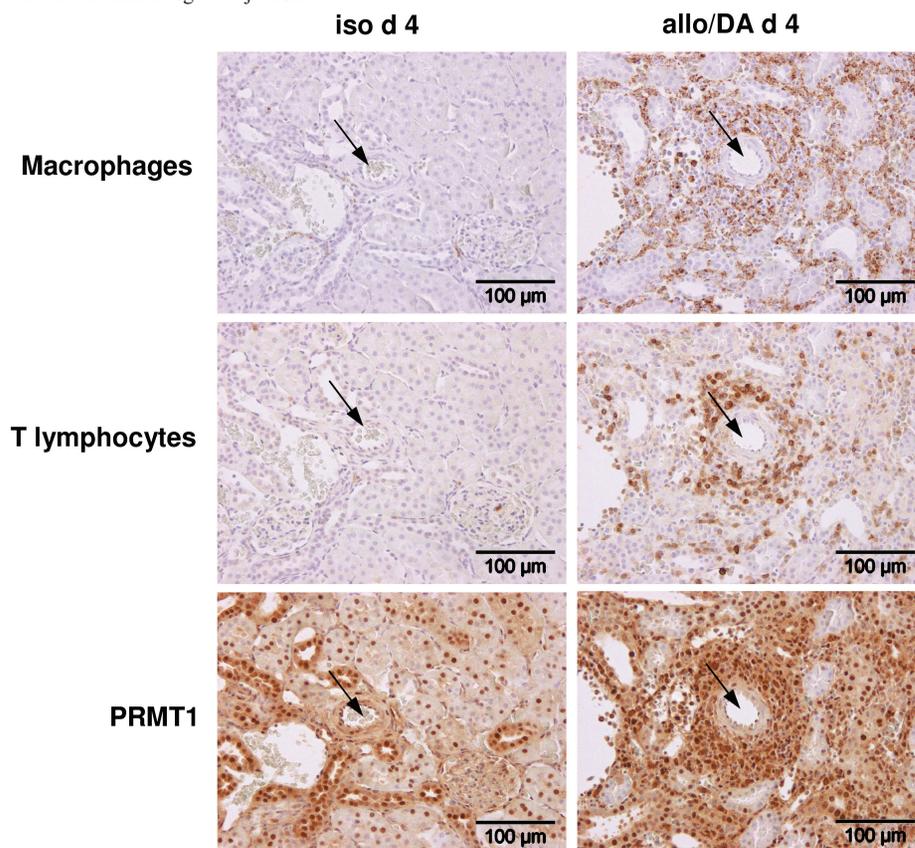
iso d 4   allo/DA d 4

Macrophages

T lymphocytes

PRMT1

Fig. 4. Localization of PRMT1 in renal isografts and allografts. Immunohistochemistry using antibodies to PRMT1, a CD68-like antigen (macrophages), and the β-chain of the α/β T-cell receptor (T lymphocytes) was performed on paraffin sections of renal isografts (iso), DA allografts (allo) on Day (d) 4 post-transplantation. Immunopositive structures were stained in brown, and the sections were lightly counter-stained with hemalum. Arrows are pointing to small arteries. Note the strongly PRMT1-immunoreactive infiltrate consisting of macrophages and T lymphocytes in the perivascular region as well as in the interstitium of renal allografts. The micrograph is representative for at least three independent experiments.

however, be argued that they are not of functional relevance. Further interventional studies would answer these questions. Certainly, more studies are needed to understand the relevance of the vigorous changes in PRMT1 expression and protein methylation during organ rejection. Increased ADMA and SDMA levels like those seen early after transplantation may interfere with the cellular uptake of L-arg by impairing NOS activity and renal re-uptake of L-arg [6]. During fatal but not during reversible acute rejection, systemic L-arg levels are reduced. As iNOS protein is strongly expressed concomitantly, probably more L-arg is consumed. Increased circulating ADMA inhibits NOS activity directly [22]. However, this does not seem to happen in our experimental setting: although dimethylarginine levels are increased in Day 4 allograft recipients, NO_x levels are elevated in plasma and in graft tissue, suggesting that iNOS is at least partially functional. Furthermore, on Day 9 after allogenic transplantation, when ADMA levels are increased in comparison to Day 42, plasma and tissue levels of NO_x are in the same range.

Possibly, high L-arg levels enable NOS function in spite of increased ADMA. It is indeed a matter of debate whether modest changes in dimethylarginine levels modulate NO production [43,44]. Previously, Cardonuel *et al.* [43] demonstrated *in vitro* that the L-arg/dimethylarginine ratio must at least decrease to 10 to elicit a physio-

logical effect, and experiments *in vivo* demonstrated that elevated ADMA levels do not necessarily result in impaired NO production [45,46]. We observe L-arg/ADMA and L-arg/SDMA ratios above 100, suggesting that NOS is unimpaired. However, ADMA and SDMA may contribute to acute renal allograft rejection by NOS-independent mechanisms such as stimulation of monocyte adhesion [5,47] and production of reactive oxygen species [5,48].

The modulation of systemic dimethylarginine levels during acute rejection is a complex process (Supplementary Figure 5). Systemic dimethylarginine concentrations are regulated by at least four variables: (i) renal function [30], (ii) ADMA degradation by DDAHs [4], (iii) protein methylation by PRMTs [29] and (iv) degradation of methylated proteins [49].

(i) Renal dysfunction may contribute to increased dimethylarginine levels in graft recipients, which are totally nephrectomized resulting at least in a 50% reduction of the functional renal mass. Graft function is indeed impaired in all recipients, but as expected, isograft function on Day 9 and 42 is superior to allograft function. Clinical studies demonstrated that ADMA and SDMA levels are elevated in patients with renal disorders [23,24,50], and dimethylarginine levels are known markers of renal function [1,51,52].
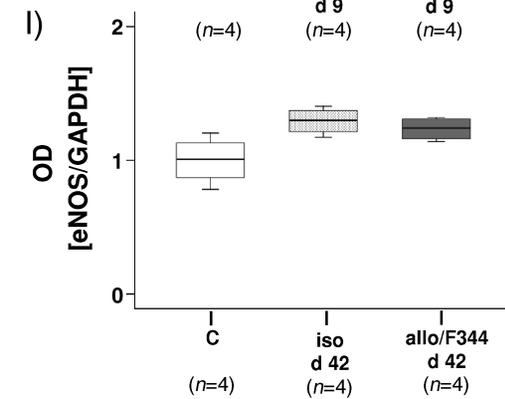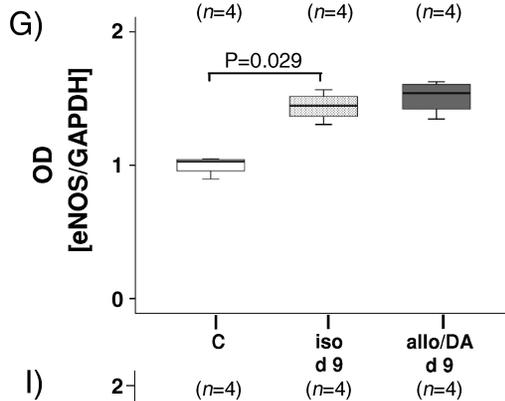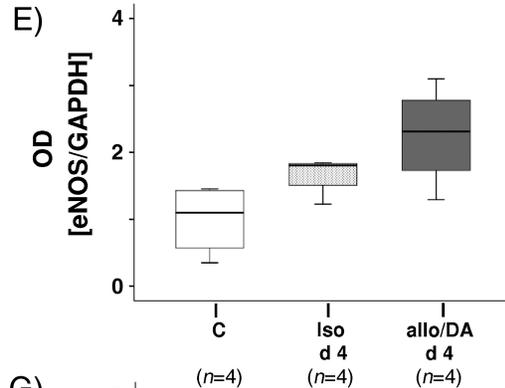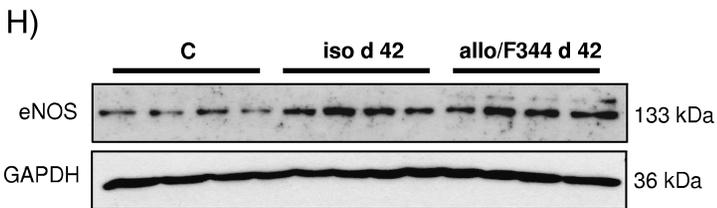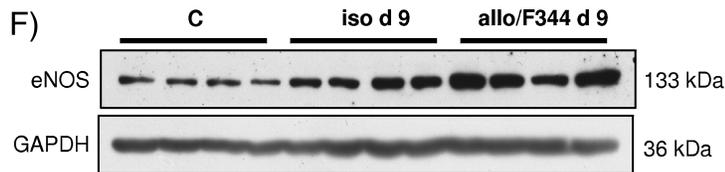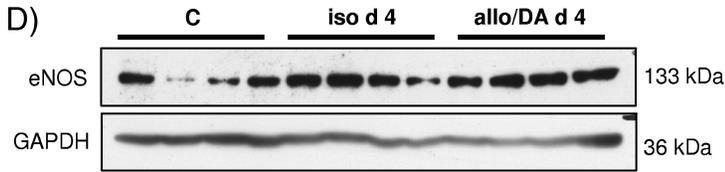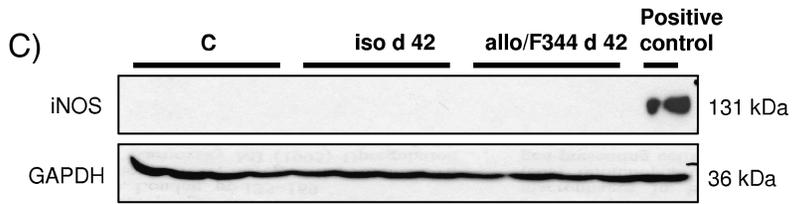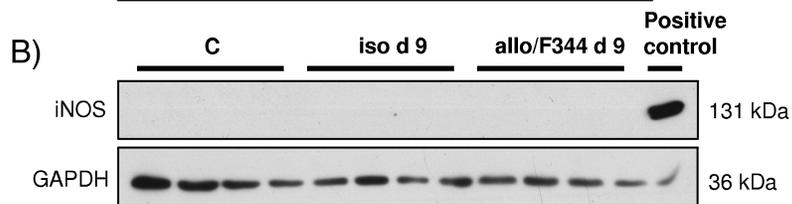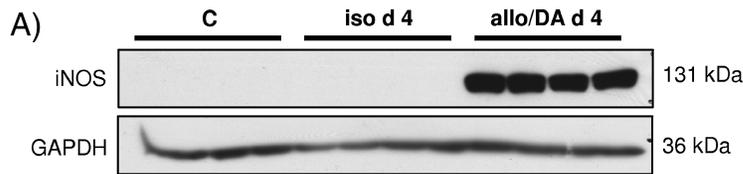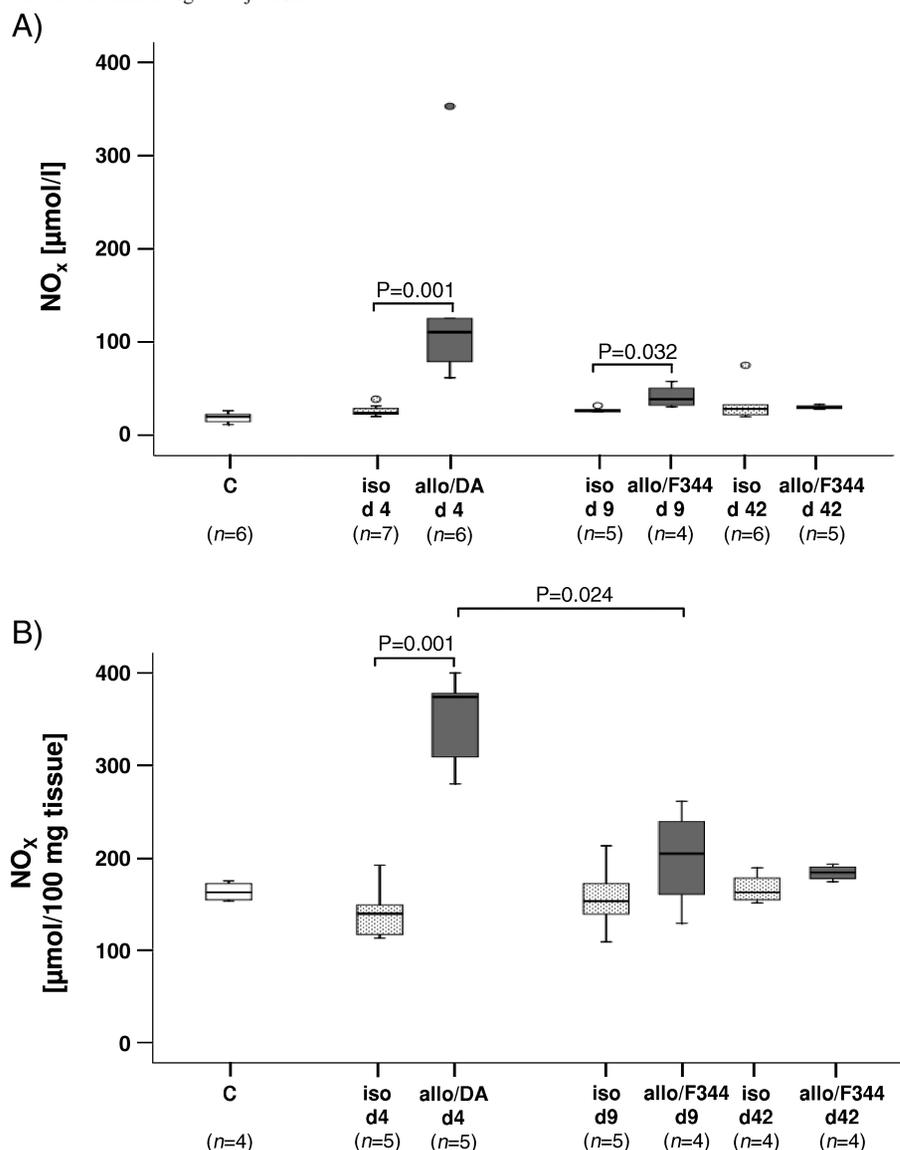
Fig. 6. NO_x concentration in plasma and renal tissue. Rat plasma (**A**) or renal tissue extracts (**B**) were subjected to Griess reaction. Untreated healthy control rats (C, white), isograft recipients (iso, dotted) and allograft recipients of DA and F344 kidneys (allo, grey) were investigated. Plasma and grafts were investigated on Day (d) 4, 9 and 42 post-transplantation. Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); circles indicate data beyond 3 × standard deviation; P-values ≤0.05 and number of animals (*n*) used in the study are indicated.

(ii) ADMA, but not SDMA, is degraded by DDAHs [32,53]. As ADMA is enzymatically degraded in the kidney [54], we analysed DDAH expression and function in graft tissue. DDAH2 mRNA slightly decreased during fatal acute rejection, which was, however, not reflected in DDAH function. In contrast, early after ischaemia/reperfusion injury of rat kidneys, total DDAH function is impaired [46]. Our data suggest that DDAH function is restored within 4 days. We conclude that changes in ADMA levels are not

due to impaired renal degradation. DDAH activity, however, may change in other organs such as the liver.

(iii) PRMT1, the dominating enzyme resulting in asymmetric protein methylation [55], is increased in all allografts. Immunohistochemistry suggests that this is due to infiltrating leucocytes expressing high PRMT1 levels. This notion is also supported by western blot experiments revealing a much stronger expression of PRMT1 in normal rat spleens compared with kidneys (data not shown). Hence, increased PRMT1 expres-

Fig. 5. NOS protein expression. Homogenates of healthy control kidneys (C, white), isografts (iso, dotted), and allografts of DA and F344 donors (allo, grey) were separated by SDS–polyacrylamide (8%) gel electrophoresis. Grafts were investigated on Day (d) 4 (**A**, **D**), 9 (**B**, **F**) and 42 (**C**, **H**) post-transplantation. Western blots were performed using specific antibodies to iNOS (A–C) and eNOS (D–H). The positive control for iNOS expression is a Day 4 DA allograft (B, C). The intensity of the resulting bands was quantified by densitometry and divided by the values obtained for the house-keeping protein GAPDH (E, G, I). The mean of the eNOS/GAPDH ratio of the controls was set to 1, and each individual value including the control values was calculated accordingly (E, G, I). Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); P-values ≤0.05 and number of animals (*n*) used in the study are indicated.

sion in allografts may contribute to increased systemic ADMA levels. Changes in renal PRMT1 expression, however, cannot be responsible for increased ADMA concentrations in Day 4 isograft recipients. In addition to PRMT1, other enzymes involved in protein methylation remain to be investigated. Changes in the methylation of several yet unindentified proteins were detected in western blots using antibodies binding to dimethylarginine–glycine repeats [42]. Methylation of proteins regulates their function [28], which is probably the most important role of PRMT. For instance, cytokine gene expression by effector T lymphocytes critically depends on methylation of the signalling molecule NIP45 [56]. Interestingly, methylation of several proteins was almost absent in Day 42 allografts.

(iv) Dimethylarginines are released upon degradation of methylated proteins [49]. It can be predicted that tissue damage during surgery results in increased protein degradation. Accordingly, ADMA and SDMA levels are increased early after transplantation. To clarify the impact of tissue damage on dimethylarginine levels, an additional group of sham-operated animals could be included. We refrained from these experiments as the data of isograft recipients, which do not develop nephropathy, suggest that the initial increase in dimethylarginine levels is not responsible for allograft nephropathy. Furthermore, allograft rejection should result in an increased turnover of leucocytes, another mechanism which may increase dimethylarginine levels.

Interestingly, ADMA and SDMA plasma levels, as well as PRMT1 and eNOS protein concentrations, were increased in isograft recipients in comparison to controls. Several factors may contribute to these differences: ischaemia/reperfusion injury caused by transplantation [46], reduced renal function due to total recipient nephrectomy or due to renal growth induced by a reduction of the total renal mass in recipient rats.

In conclusion, our data do not support the concept that the fate of renal allografts during acute rejection is decided by dimethylarginines. Dimethylarginines may play a role in triggering chronic rejection, but a contribution to the process of vascular remodelling itself is improbable. In contrast, differential arginine methylation by PRMT1 may be involved in the pathogenesis of acute and chronic rejection.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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