

cAMP Pulse During Preservation Inhibits the Late Development of Cardiac Isograft and Allograft Vasculopathy

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Abstract—The causes of transplant-associated coronary artery disease remain obscure, and there is no known treatment. Preservation injury of murine heterotopic vascularized cardiac isografts caused a small, albeit significant, increase in neointimal formation; preservation injury of allografts markedly increased both the incidence and severity of transplant-associated coronary artery disease. As cAMP is an important vascular homeostatic mediator the levels of which decline during organ preservation, buttressing cAMP levels solely during initial preservation both improved acute allograft function and reduced the severity of transplant-associated coronary artery disease in grafts examined 2 months later. Inhibiting the cAMP-dependent protein kinase abrogated these beneficial effects. cAMP treatment was associated with an early reduction in leukocyte infiltration and a reciprocal decrease in superoxide and increase in NO levels. These data indicate that alloantigen-independent injury to the graft, which occurs at the time of cardiac preservation, can set in motion pathological vascular events that are manifest months later. Furthermore, a cAMP pulse during cardiac preservation reduces the incidence and severity of transplant-associated coronary artery disease. (*Circ Res.* 2000;86:982-988.)

Key Words: cAMP ■ protein kinase ■ heart transplantation ■ allograft arteriopathy ■ organ preservation

Although cardiac transplantation is a life-saving procedure for patients who would otherwise die from intractable heart failure, ≈50% of all recipients of heart transplants develop cardiac transplant-associated coronary artery disease (TCAD) by 5 years after transplantation.¹ The progression of TCAD in human heart transplant recipients is inexorable, with no effective therapy short of retransplantation. Identification of the precise etiologic factors leading to TCAD remains elusive. It is believed that TCAD may be related to a donor-specific, cell-mediated alloreactivity to donor vascular endothelium.² Although immunologic disparity between donor and recipient undoubtedly exacerbates TCAD,³ in addition to immune system-activating mechanisms,⁴ there remains the possibility that antigen-independent factors can also contribute to or accelerate the progression of TCAD.

In clinical studies, pinpointing a causal role for ischemia in TCAD development has been elusive, although there is a significant amount of circumstantial data suggesting a role for ischemic injury in the evolution of human TCAD. In a large study by Opelz and Wujciak,⁵ in which >8000 cardiac transplant recipients were examined, a clear relation was seen between the duration of cold ischemia and graft survival as long as 3 years after cardiac transplantation (TCAD was not specifically examined). In fact, when the duration of cold

ischemia was 6 hours, graft survival by 3 years out was nearly 25% less than those grafts preserved under hypothermic conditions for <2 hours. In this study, intermediate preservation durations were associated with intermediate durations of graft survival. Several previous studies have suggested that cold ischemic injury accelerates the progression of both acute and chronic rejection.^{6,7} In a study by Hosenpud et al.,⁸ longer donor cold ischemic times were also associated with a higher mortality at 1 year than recipients whose grafts were preserved for shorter durations. In a case-control study,⁹ endomyocardial biopsy specimens were graded histologically for the degree of ischemic injury, and a multivariate analysis was performed to determine which of a number of variables could predict the subsequent development of TCAD. Of the variables examined, including the number of major histocompatibility complex (MHC) class I mismatches, donor age, recipient cytomegalovirus status, number of rejection episodes in the first year, and histologic degree of ischemic injury, degree of ischemic injury emerged as the strongest predictor for the subsequent development of TCAD. The current studies were designed to test the hypothesis that ischemia-reperfusion injury is by itself sufficient to induce TCAD and that, in an allograft milieu, early ischemia reperfusion injury is a powerful accelerant and exacerbating factor for TCAD development.

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Materials and Methods

Experimental Animals and Graft Preservation

Male B10A(H2a) mice were used as donors, and C57BL/6J(H-2b) mice were used as recipients. The base preservation solution consisted of a buffered electrolyte solution either alone or with the following additional reagents added: *N*⁶,2'-*O*-dibutyryladenosine 3':5'-cyclic monophosphate (db-cAMP); 8-bromoadenosine-cAMP (8-Br-cAMP); and 8-Br-cAMP+Rp-cAMPS, the Rp isomer of adenosine 3',5'-monophosphothioate.

Experiments were performed according to a protocol approved by the Columbia University Institutional Animal Care and Use Committee and are in accordance with AAALAC guidelines.

Immunosuppression and Transplantation

Transient immunosuppression was performed by preoperative administration of anti-murine CD4 and anti-CD8 antibodies at days 6, 3, and 1 before the transplantation.¹⁰ Heterotopic cardiac transplantation was performed as described in a previous study.¹¹

Graft functional assessment was performed using a scoring system developed in a previous study^{12,13} at 30 minutes after reperfusion and by manual palpation every other day during the 60-day observation period (0 to 3; 3=strongest contraction). At 60 days (or at the time that contraction ceased), the graft was harvested. Two-dimensional echocardiographic assessment of cardiac graft function was performed at 24 hours after transplantation. Instrumentation and methods to measure fractional area change are described in the expanded online Materials and Methods (<http://www.circresaha.org>).

Quantification of TCAD Area

Histomorphometry of TCAD area on elastin-stained sections was performed using techniques similar to those reported by others.^{14–16} Planimetered areas were calculated by image-analysis software. Percentage of luminal obliteration (TCAD area, %) was determined as described in the expanded online Materials and Methods (<http://www.circresaha.org>).

Graft leukocyte accumulation was quantified using a chromogenic assay to detect the enzyme myeloperoxidase¹⁷ 24 hours after reperfusion.

NO/Superoxide Measurement

NO was measured by chronoamperometry using a catheter-protected porphyrinic microsensor.¹⁸ O₂⁻ was measured using the chemiluminescence method.¹⁹ Specifics of measurement and calibration techniques are described in the expanded online Materials and Methods (<http://www.circresaha.org>).

Data Analysis

Myeloperoxidase activity, transplantation scores, and palpation scores were compared using the Mann-Whitney *U* test for unpaired variables. Other data were analyzed using ANOVA. Results are expressed as mean±SEM. Statistical significance was defined as *P*<0.05.

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

Results

The role of early vascular injury in the late development of TCAD was studied in a murine heterotopic cardiac transplant model.¹¹ Using this model, a brief preoperative period of immunosuppression was used to prevent acute rejection; however, over the ensuing several weeks to months, diffuse concentric lesions develop within the coronary vascular tree which are similar to human TCAD in that, when advanced, they may completely obliterate the vascular lumen.^{10,20} Unlike previous reported experiments,^{20,21} the current experiments were performed with a variable cold preservation duration, to establish whether increased preservation duration

increased neointimal formation in this model. Allograft experiments, performed using B10.A(2R) donor hearts transplanted into C57BL/6J recipient mice, showed that with meticulous operative technique and essentially immediate transplantation after harvest (without an intervening *ex vivo* period of cold preservation), neointimal formation at 2 months was often absent (only 46% of grafts that were immediately transplanted demonstrated TCAD). In these nonpreserved allografts, on average, the neointima accounted for 15.7±6.2% of the luminal obliteration at the 60-day observation point (Figure 1A).

When isograft experiments were performed with similar immediate transplantation without an intervening cold preservation period, little if any neointimal formation was observed (TCAD area, 4.4±1.3%, consistent with previous reports in the murine heart transplant model⁴ [Figure 1G]). Using the 120-minute preservation period as a test condition for isografts, at the 2-month observation point, a small but significant increase in neointimal area was observed (8.1±1.8%) compared with nonpreserved isograft controls (Figure 1H). These data indicate that, even in the absence of an alloimmune response, preservation injury can promote some TCAD development.

In contrast to these data gathered in isografts, cardiac allografts subjected to long cold preservation times exhibited a striking increase in both the incidence and severity of TCAD. When allografts were subjected to 90 minutes of cold preservation, the neointima accounted for 50.2±10.2% luminal obliteration at the 60-day observation point (Figure 1B). Increasing the cold preservation duration to 120 minutes resulted in an even greater increase in TCAD area (Figure 1C). To more fully characterize the relationship between cold ischemic duration, graft injury, and delayed TCAD development, allograft function was scored 30 minutes after transplantation using a previously described scoring system,^{12,13} in which cardiac contractility, turgor, and color were judged by an observer blinded to the preservation conditions. These transplant score data were recorded for each of the cold preservation times. Not surprisingly, increased cold preservation duration was associated with increased graft injury (Figure 2A). When these early transplant score data were then correlated with TCAD area determined 2 months later at euthanasia, the data show a striking inverse linear correlation between initial transplant score and mean lesional cross-sectional area determined by histomorphometry (Figure 2B).

Because we have previously shown that ischemia-driven cAMP deficiency in preserved organs is a critical mechanism by which vascular homeostasis is disrupted,^{12,22} we hypothesized that replenishing cAMP at the time of preservation would not only improve acute graft function, but inhibit delayed TCAD development. In allografts preserved for 120 minutes of cold ischemia, adding the membrane-permeable cAMP analog db-cAMP to the flush/preservation solution resulted in both early functional improvement and markedly diminished TCAD formation at 2 months. Thirty minutes after reperfusion, transplant scores were significantly higher in db-cAMP-supplemented grafts than in controls (Figure 2A).

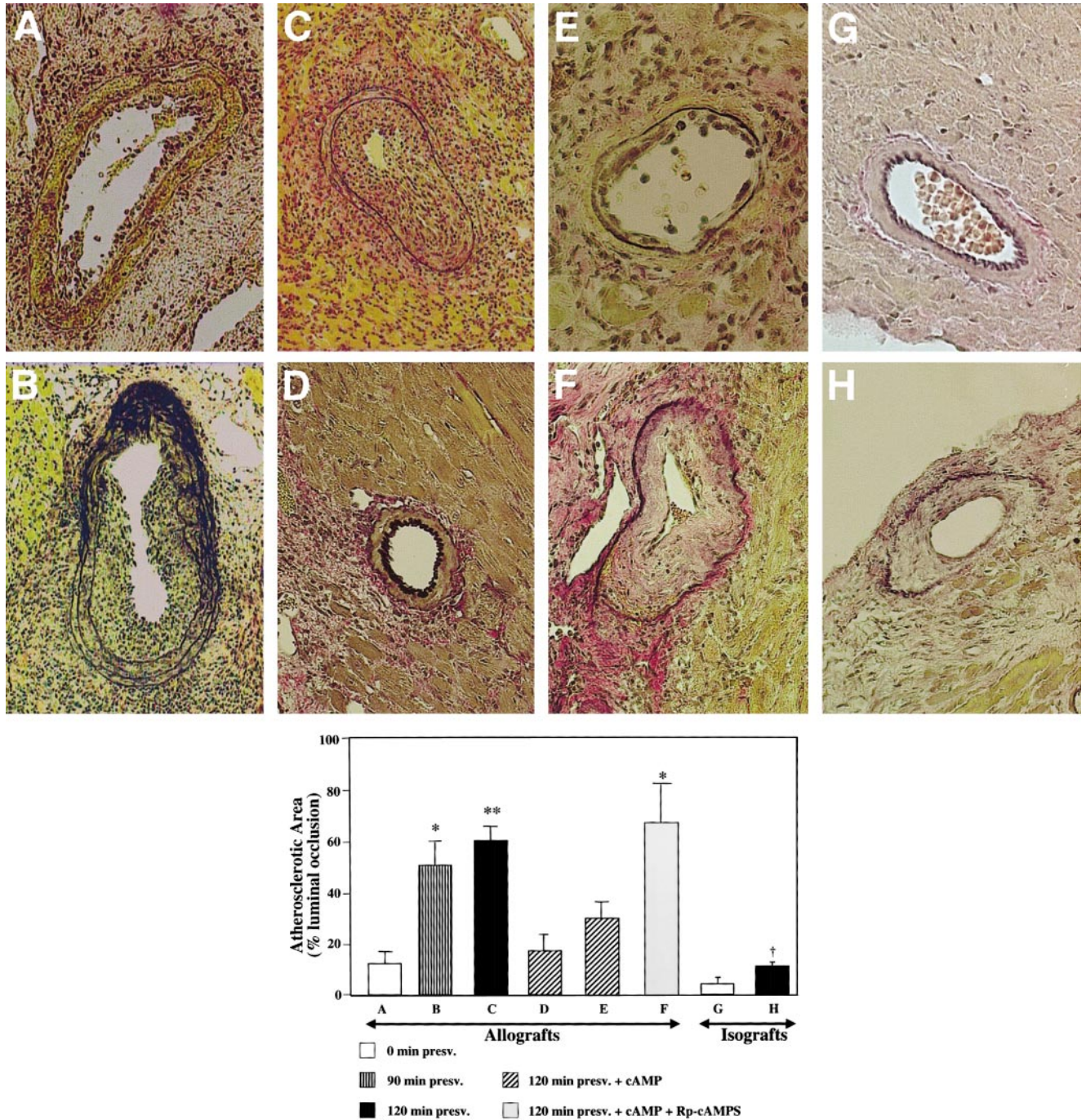


Figure 1. Effect of preservation duration on late development of TCAD and role of the cAMP second messenger pathway in TCAD development in the murine heart transplant model. Top, Representative vessels from allografts (A through F) or isografts (G and H) harvested 60 days after transplantation. A, Vessel from a graft that had been transplanted immediately after harvest, without an intervening hypothermic preservation period. B and C, Vessels from allografts subjected to 90-minute (B) or 120-minute (C) hypothermic preservation before implantation, without preservation solution additive. D and E, 120-minute preservation with added cAMP analog db-cAMP (2 mmol/L) (D) or 8-Br-cAMP (0.1 mmol/L) (E). F, 120-minute preservation, with 8-Br-cAMP (0.1 mmol/L) and the cAMP-dependent protein kinase inhibitor Rp-cAMPS (0.25 mmol/L) added to the flush/preservation solution. G, Vessel from an isograft that had been subjected to 0 minutes of preservation. H, Vessel from an isograft that had been subjected to 120 minutes of preservation. Bottom, Degree of TCAD was objectively quantified histomorphometrically using a computer-based imaging system. Data are mean±SEM for each group. Numbers of transplants were as follows: A, 13; B, 5; C, 6; D, 12; E, 7; F, 4; G, 5; and H, 6. Mean number of vessels analyzed for each section was 17±5.

Improved graft function by db-cAMP supplementation was confirmed by transabdominal 2-dimensional echocardiography of the transplanted hearts performed at 24 hours after transplantation. These studies showed that fractional area

change was increased 3.6-fold in db-cAMP-preserved grafts compared with control grafts (Figure 2C). When these same grafts were assessed for TCAD development after 2 months of observation, there was a marked (3.4-fold) diminution in

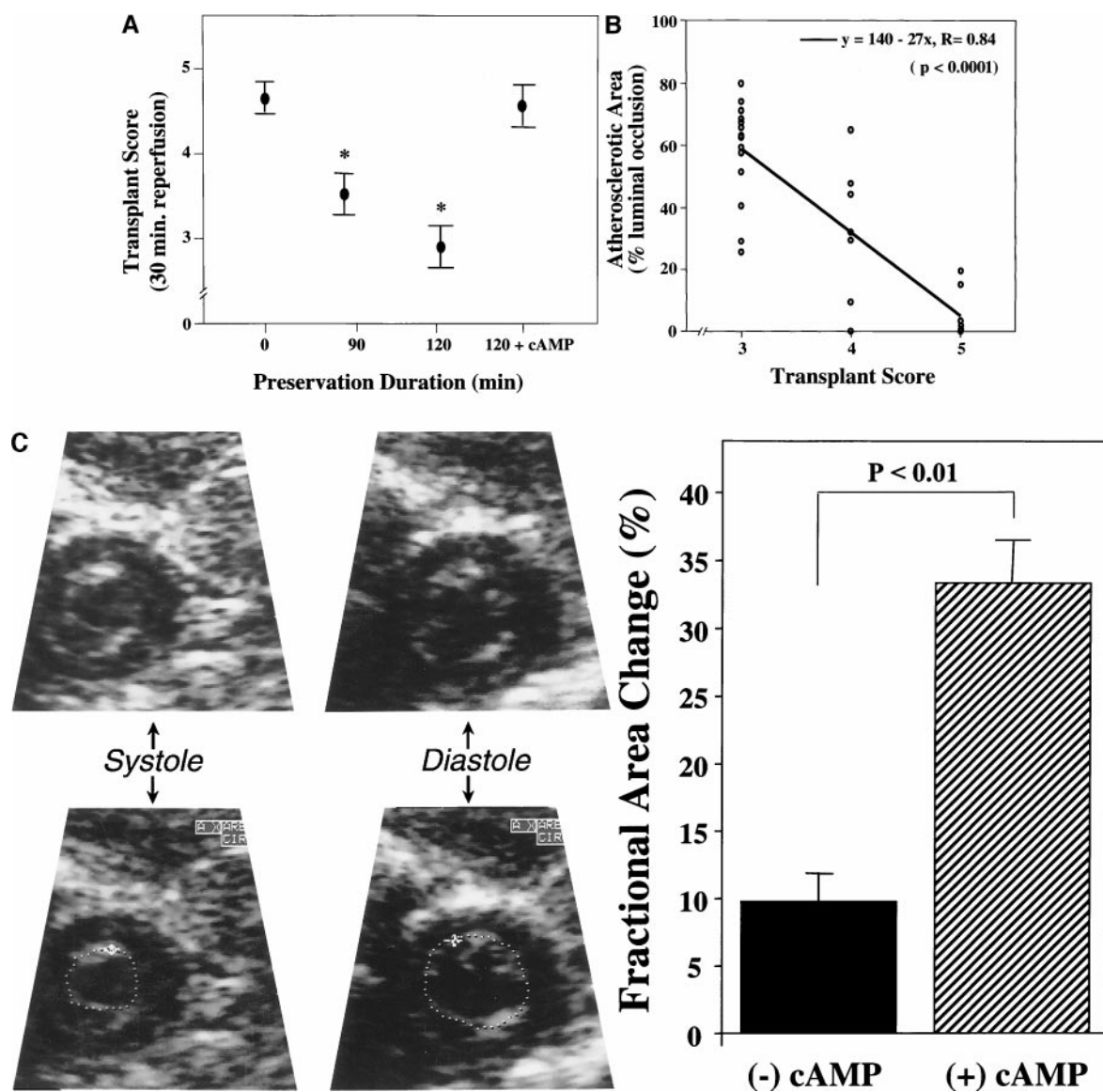


Figure 2. Effect of early ischemic injury on allograft function and late TCAD development. A, Using a scoring system described previously,^{12,13} cardiac grafts were assessed by a blinded observer on the basis of contractility (0 to 2, 2=best), color (0 to 2, 2=pink), and turgor (0 to 1, 1=soft) at 30 minutes after transplantation. B, Relationship between early ischemic injury (transplant score at 30 minutes) and TCAD in cardiac allografts subjected to 120 minutes of hypothermic preservation in the absence of a preservation solution additive. Each point represents a single transplant experiment, which gives a single transplant score and a single mean TCAD area. C, In vivo echocardiographic imaging of murine cardiac allografts 24 hours after transplantation. Left, Freeze-frame images at end systole and end diastole are shown, with the endocardial contours indicated with a dashed line. Right, Fractional area changes (%) were calculated for each group and are expressed as mean \pm SEM. $n=6$ for (-) cAMP, and $n=5$ for (+) cAMP.

TCAD area (Figure 1D) compared with grafts preserved in the absence of cAMP analog (Figure 1C), despite the identical 120-minute preservation time.

cAMP supplementation has been shown to improve early function of lung isografts by inhibiting leukocyte influx, diminishing edema formation, and reducing platelet accumulation.²² To investigate potential mechanism(s) by which db-cAMP may be acting during the early post-transplant period that may influence delayed TCAD development, graft neutrophil infiltration was examined in a separate cohort of 120 minute-preserved grafts stored in the presence or absence of db-cAMP. Inclusion of db-cAMP in the cardiac preservation solution in these experiments diminished early graft leukocyte infiltration, quantified by graft myeloperoxidase activity (which measures pri-

marily granulocytes, but also can also be observed at lower levels in mononuclear phagocytes^{23,24} [Figure 3A]). As recruited leukocytes undergoing the respiratory burst generate superoxide anion in the reperfusion milieu, we next investigated the effects of cAMP-buttressed preservation on superoxide levels in grafts (as well as NO, which is quenched by superoxide). A separate cohort of animals was transplanted, and at 16 hours, their hearts were subjected to NO measurements performed in vivo using a porphyrinic microsensors,¹⁸ as well as superoxide measurements in explanted graft tissue. Supplementation of the preservation solution with db-cAMP resulted in a normalization of NO levels in concert with a suppression of superoxide levels detected by chemiluminescence (Figures 3B and 3C). Normalization of NO levels may be a mechanism by which cAMP supplementation

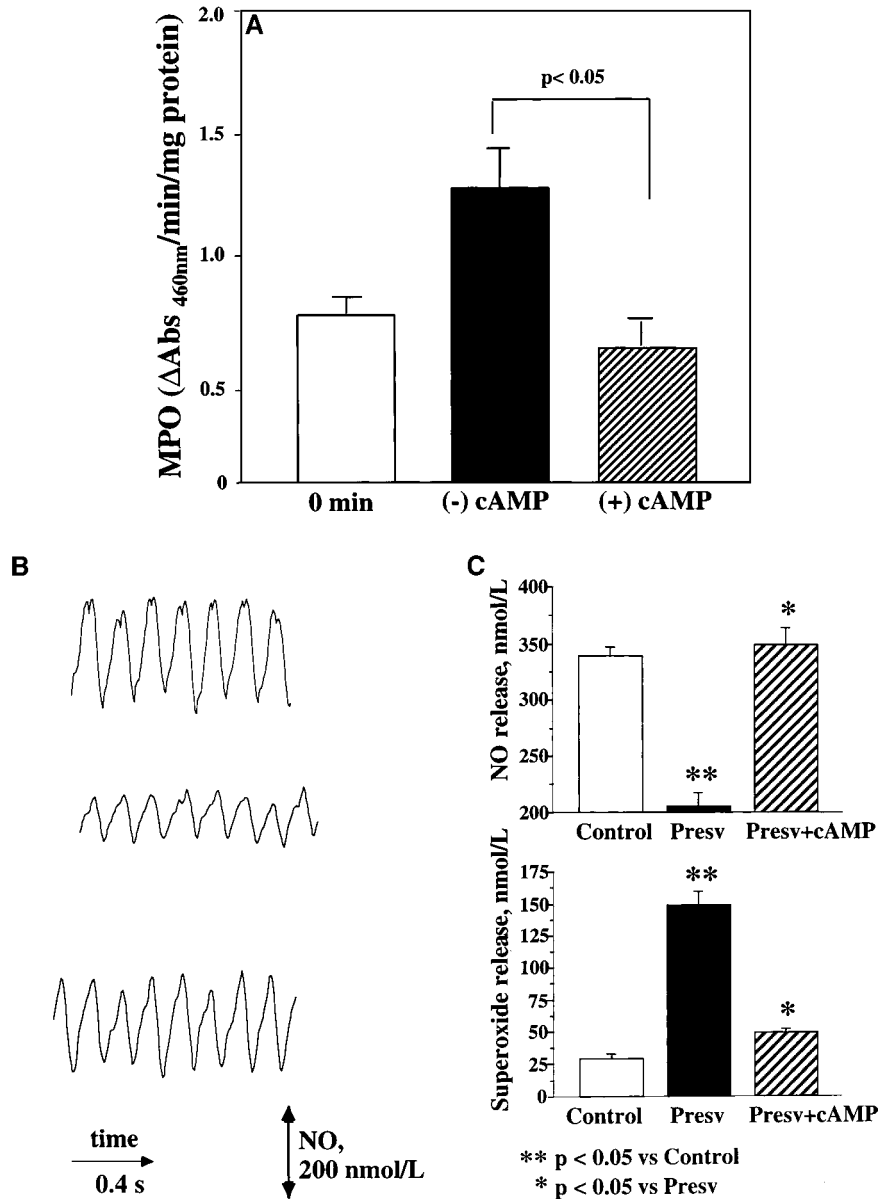


Figure 3. Effect of cAMP supplementation on graft neutrophil infiltration, NO, and superoxide levels. Grafts were transplanted immediately after harvest (0 minutes of preservation) or preserved for 120 minutes in the absence (-cAMP) or presence (+cAMP) of db-cAMP (2 mmol/L). A, Effect of cAMP on graft neutrophil accumulation measured at 24 hours after transplantation, quantified by measuring myeloperoxidase activity (MPO). Data are mean \pm SEM for each group. n=6 for controls, and n=7 for the cAMP-treated group. B, In vivo measurement of NO in the beating/transplanted mouse heart measured at 16 hours after transplantation, using a porphyrinic microsensor that is sensitive and specific for NO detection. Top, Graft was harvested in preservation solution without additive and immediately transplanted (0 minutes of preservation). Middle, Graft was harvested in preservation solution without additive and transplanted after 120-minute preservation (corresponds to Presv in panel C). Bottom, Graft was harvested in preservation solution supplemented with db-cAMP (2 mmol/L) and transplanted after 120-minute preservation (corresponds to Presv+cAMP in panel C). C, Quantification of peak NO concentration (top) by porphyrinic sensor and superoxide levels (bottom) obtained using the chemiluminescent method described in Materials and Methods. Experiments were performed as described in panel B.

acts to inhibit delayed TCAD development, as NO synthase II has been shown to have a protective role against the development of TCAD.²⁵

To demonstrate that the beneficial effects of db-cAMP were not specific for this cAMP analog and accrue through activation by cAMP of the cAMP-dependent protein kinase, an additional series of experiments was performed. Incorporation of the membrane-permeable cAMP analog 8-Br-cAMP into the flush/preservation solution caused a similar reduction of TCAD area at 2 months (Figure 1E). When the same dose (0.1 mmol/L) of 8-Br-cAMP was added to the preservation solution concomitant with addition of the cAMP-dependent protein kinase inhibitor, Rp-cAMPS,²⁶ the beneficial effects of the cAMP analog with respect to diminishing TCAD were completely lost (Figure 1F). Although these experiments do not preclude an additional protective role that may be conferred by NO-mediated stimulation of the cGMP-dependent protein kinase, it is unlikely for db-cAMP to enhance preservation by direct activation of the

cGMP-dependent protein kinase for the following reason. *N*⁶-Monobutyryladenine 3',5'-monophosphate (the active compound formed after db-cAMP enters the cell)²⁷ and 8-Br-cAMP are, respectively, 313-fold and 53-fold less potent than cGMP in activating the cGMP-dependent protein kinase.^{28,29} An indirect role is possible, however, given that reduced leukocyte recruitment, reduced superoxide levels, and increased NO levels were detected in cAMP-supplemented grafts.

Discussion

These data demonstrate several important features of TCAD, as studied in the murine model of heart transplantation. First, although an alloimmune response significantly increases the degree of TCAD development, some TCAD may be observed in the absence of an alloimmune barrier. There are suggestions in the literature that ischemic injury can exacerbate acute or chronic rejection.^{6,7,30} The data shown here are the first to address this issue in a systematic fashion specifically

in the murine model of cardiac transplant vasculopathy and show a correlation between ischemic preservation duration and development of TCAD. A second important aspect of these data is also apparent, that in the presence of an alloimmune barrier, preservation/reperfusion injury can significantly exacerbate TCAD development, even months after the transplant procedure. An implication of these data is that improving the early preservation or reperfusion milieu might reduce the incidence or severity of TCAD in human heart transplantation (this is a hypothesis that remains to be tested). Even if remotely true, however, this would represent an important advance, as there are currently no accepted treatment modalities, and preventive measures would be welcome. There is a third important point that can be gleaned from the current data. Stimulating the cAMP-dependent protein kinase, which we have previously shown to benefit the immediate (10-minute) post-transplantation vascular milieu,¹² has delayed beneficial effects to reduce the severity and incidence of TCAD, even in grafts that should have sustained more ischemic injury (ie, with extended preservation durations). These experiments go further, to suggest that one of the potential ways in which protein kinase A stimulation may be beneficial is by reducing graft oxidant stress and restoring NO levels. This is not likely to be the only mechanism by which stimulating this pathway may be beneficial. Independent of its effects on NO, cAMP has known effects to modulate leukocyte adhesion, graft edema, thrombosis, and vascular tone.

Although this study does not directly address the mechanism by which cAMP reduces leukocyte adhesivity to endothelium, there is a substantial body of literature on the subject. cAMP may diminish polymorphonuclear leukocyte (PMN) adhesiveness by elevating intracellular calcium within PMNs, inhibiting mobilization and surface expression of the PMN β_2 integrin CD11b/CD18, as well as shape change of the PMN.³¹ cAMP may also have an anti-leukocyte-adhesive action because of specific effects on endothelial cells, with decreased synthesis of E-selectin and vascular cell adhesion molecule-1 when intracellular cAMP levels are elevated.³²

There are a number of potential mechanisms whereby ischemia/reperfusion injury might increase the incidence or severity of chronic rejection. Any stimulus that increases immunogenicity of the donor vasculature is a prime suspect for accelerating TCAD development. For instance, even short periods of ischemia have been shown to upregulate the expression of class I and II MHC antigens. In a mouse model of renal ischemia, there was a 3- to 6-fold increase in class I antigen expression and an approximate doubling of MHC class II antigen expression.³³ In a rat model of unilateral lung ischemia, the ischemic period led to a marked increase in MHC class II molecule expression, which was especially pronounced in the presence of allogeneic leukocytes.³⁴ In other renal transplant settings, recovery from ischemic injury has likewise been shown to increase the expression of MHC class I and II antigens.³⁵ These data, along with the observation that ischemia at the time of transplantation correlates with the subsequent incidence of reversible rejection episodes in both renal³⁶ and hepatic transplantation,³⁷ suggest that grafts subjected to prolonged ischemia are more immuno-

genic than those in which ischemia/reperfusion injury is less pronounced. Another potential mechanism to explain the increased immunogenicity of grafts that have experienced significant ischemia is that ischemic injury can result in increased release of donor endothelial antigens at the time of transplantation. This could explain an association that has been noted between the appearance of endothelial cell antibodies and rapid progression of TCAD (unassociated with cellular rejection).³⁸ Other mechanisms of vascular injury, such as complement activation, which occurs secondary to ischemia^{39,40} or after hypothermic preservation and organ transplantation,⁴¹ or disruption of the fibrinolytic/anticoagulant balance in ischemic vasculature,⁴² may also play a role in ischemia-exacerbated TCAD.

The data presented here strongly support an oxidant injury-induced mechanism of alloantigen-independent TCAD development, as grafts with prolonged ischemia demonstrated increased O_2^- levels and reduced NO levels. The lucigenin method for detecting O_2^- has been advocated by many; however, there is some residual controversy over its use.⁴³⁻⁴⁶ Nevertheless, there is substantial evidence that superoxide is generated during cardiac reperfusion, which is consonant with the data presented here with the lucigenin method of detection. Although the acute failure of the NO pathway has been previously reported to result in vascular compromise within minutes after transplantation of either the heart¹³ or the lungs,⁴⁷ these data show that there are long-term adverse consequences to early graft vascular failure.

Taken together, the data presented show that cold ischemic injury promotes the late development of TCAD, which can occur even in the absence of an alloimmune response. A pulse of cAMP, given by adding a membrane-permeable cAMP analog to the cardiac flush/preservation solution, reduces early leukocyte influx and superoxide levels, increases NO levels, normalizes early graft function, and virtually abrogates TCAD. The quality of preservation at the time of cardiac preservation deserves rigorous attention, because it can profoundly impact on the subsequent occurrence of TCAD. As there are no therapies for established TCAD, the studies presented here should focus attention on the early preservation and reperfusion milieus as an opportunity to take prophylactic measures against its occurrence.

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