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Decreased cADPR and increased NAD^+ in the *Cd38*$^{-/-}$ mouse ☆

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Abstract

CD38 is a type II glycoprotein that catalyzes the formation of cyclic ADP-ribose (cADPR), an intracellular calcium signalling molecule, from nicotinamide adenine dinucleotide (NAD^+). Using a modified version of the fluorimetric cycling assay for cADPR which reduces between-subject variability, we report significant decreases in brain and lung cADPR, which although similar to previously published values, showed much less individual variation. The reduced variation within each group suggests that the range of cADPR is narrower than previously thought, and that the regulatory mechanisms controlling these levels are more finely tuned. We also report significant increases in brain, lung, and kidney NAD^+ in the *Cd38*$^{-/-}$ mouse, and provide the first experimental demonstration of the proximate relationship between CD38 and NAD^+.

Keywords: CD38; cADPR; NAD^+; ADP-ribosyl cyclase; Pyridine nucleotides; Ca^{2+} signalling

CD38 is a type II glycoprotein that has dual function as both a surface antigen and a catalyst [1,2]. CD38 is expressed in many immune and hematopoietic cells, including B and T cells [3–5], monocytes [6], red blood cells [7], and platelets [8], and the protein is distributed widely in tissues such as the prostate [9], the pancreas [10], the spleen [11], the heart [12], and the brain [13,14]. Although originally described as having a solely ectocellular location, CD38 has subsequently been identified in association with intracellular membranes [14,15], where its protein structure would localize the active site of the enzyme towards the inside of the organelle or vesicle with which it was associated [16].

Many of the functions related to CD38 can be ascribed to its role as an ADP-ribosyl cyclase enzyme, generating the calcium signalling molecule cADPR from the pyridine nucleotide nicotinamide adenine dinucleotide (NAD^+) [2]. The proximate relationship between CD38, cADPR, and NAD^+ suggests that levels of these nucleotides may be modulated in response to deletion of the CD38 gene. Earlier studies [17,18] measured levels of cADPR in the *Cd38*$^{-/-}$ mouse, and found non-significant reductions in the brain, lungs, kidneys, and heart. NAD^+ levels have not been previously investigated. The present study was designed to examine the effect of deletion of the CD38 gene on levels of cADPR in the *Cd38*$^{-/-}$ mouse using a modified version of the fluorimetric cycling assay for cADPR, which we have shown reduces variability between samples collected from individual animals [19]. As well, we investigated the effect of gene deletion on levels of NAD^+, which as the substrate for CD38 is expected to be modulated in response to loss of the protein.

Materials and methods

Animals and tissue preparation. Male *Cd38*$^{-/-}$ and male C57BL6/J adult mice were obtained from the Trudeau Institute (Saranac Lake, NY). *Cd38*$^{-/-}$ mice were generated by gene targeting [20] and were backcrossed for 12 generations to C57BL/6J [18]. This study was approved by the Animal Care Committee at the University of Guelph and adhered to the

☆ *Abbreviations:* cADPR, cyclic ADP-ribose; NAD^+, nicotinamide adenine dinucleotide; PCA, perchloric acid; KOH, potassium hydroxide.

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standards set forth by the Canadian Council on Animal Care. For preparation of brain tissue, animals were anesthetized with Isofluorane (Fisher Scientific) and the heads were then submerged in liquid nitrogen for approximately 1 min. This method of termination was used in order to maintain brain energy metabolism, which requires freezing of the brain while oxygenated blood is still circulating [21,22]. Decapitation was performed with a guillotine. Heads were collected and placed immediately into liquid nitrogen and stored at −80 °C. Later, the brain was dissected out of the skull while still frozen and placed immediately into a solution of 1 M perchloric acid (PCA) at a concentration of 0.1 g tissue to 1 ml PCA. Frozen tissue was then homogenized with an electric homogenizer. Samples were centrifuged twice for 10 min, with the first at 1000*g* and the second at 14,000*g*. The aqueous layer was recovered from both centrifugations. For preparation of heart, lung, and kidney tissue, animals were euthanized with carbon dioxide and the organs were rapidly dissected and placed immediately into liquid nitrogen. Frozen tissue was then placed into a solution of 1 M perchloric acid (PCA), and the homogenization and centrifugations were performed as described above.

Measurement of cADPR. The assay used to measure levels of cADPR was that described by Graeff and Lee [23], with some modifications. In order to increase the recovery of cADPR following removal of PCA, samples were neutralized to pH between 7.7 and 7.9 using 2 M potassium hydroxide (KOH), instead of using the organic extraction described in [23]. The protocol was further modified to remove the NAD^+ hydrolytic enzymes from the treated samples and standards by acidification. After overnight incubation, the enzymes were removed by acidification with 1 M PCA (to pH between 2.5 and 2.9). Samples and standards were centrifuged at 14,000*g* and the aqueous layer was recovered and re-neutralized to pH between 7.7 and 7.9 with 2 M KOH. Another centrifugation at 14,000*g* was performed to precipitate the perchlorate salt. Standards were prepared in 1 M PCA and carried through all steps of the assay. Additionally, the diaphorase enzyme was treated at room temperature to remove contaminating NAD^+ following observation of a reduction of activity after treatment at 37 °C. The remainder of the assay was as previously described [23]. In brief, samples and standards were carried through the cycling reaction by addition of reagents including ADP-ribosyl cyclase (forced to run in reverse by high [nicotinamide]), sodium phosphate, nicotinamide, ethanol, alcohol dehydrogenase, resazurin, diaphorase, FMN, and BSA. During the cycling reaction, cADPR is converted to NAD^+ by ADP-ribosyl cyclase, and the NAD^+ is cycled to NADH by alcohol dehydrogenase. NADH is then reduced to NAD^+ by diaphorase, producing fluorescent resorufin. Samples and standards are run under duplicate conditions with both the presence and the absence of the cyclase, allowing for subtraction of signals originating from contaminating NAD^+. The cycling reaction can proceed for several hours, thereby amplifying the initial cADPR concentration by more than a thousand.

Measurement of NAD^+. Samples were diluted fivefold in PCA from the initial concentration of 0.1 g tissue to 1 ml PCA. Upon thawing, all samples were neutralized to pH between 6.7 and 6.8 with 2 M KOH and analyzed for NAD^+ content via the enzyme cycling of alcohol dehydrogenase [24].

Statistical analysis. Data are expressed as means ± standard error of the mean. Two-tailed independent *t*-tests were used to compare levels of cADPR and NAD^+ in wild type as compared to *Cd38*$^{-/-}$ mice. cADPR

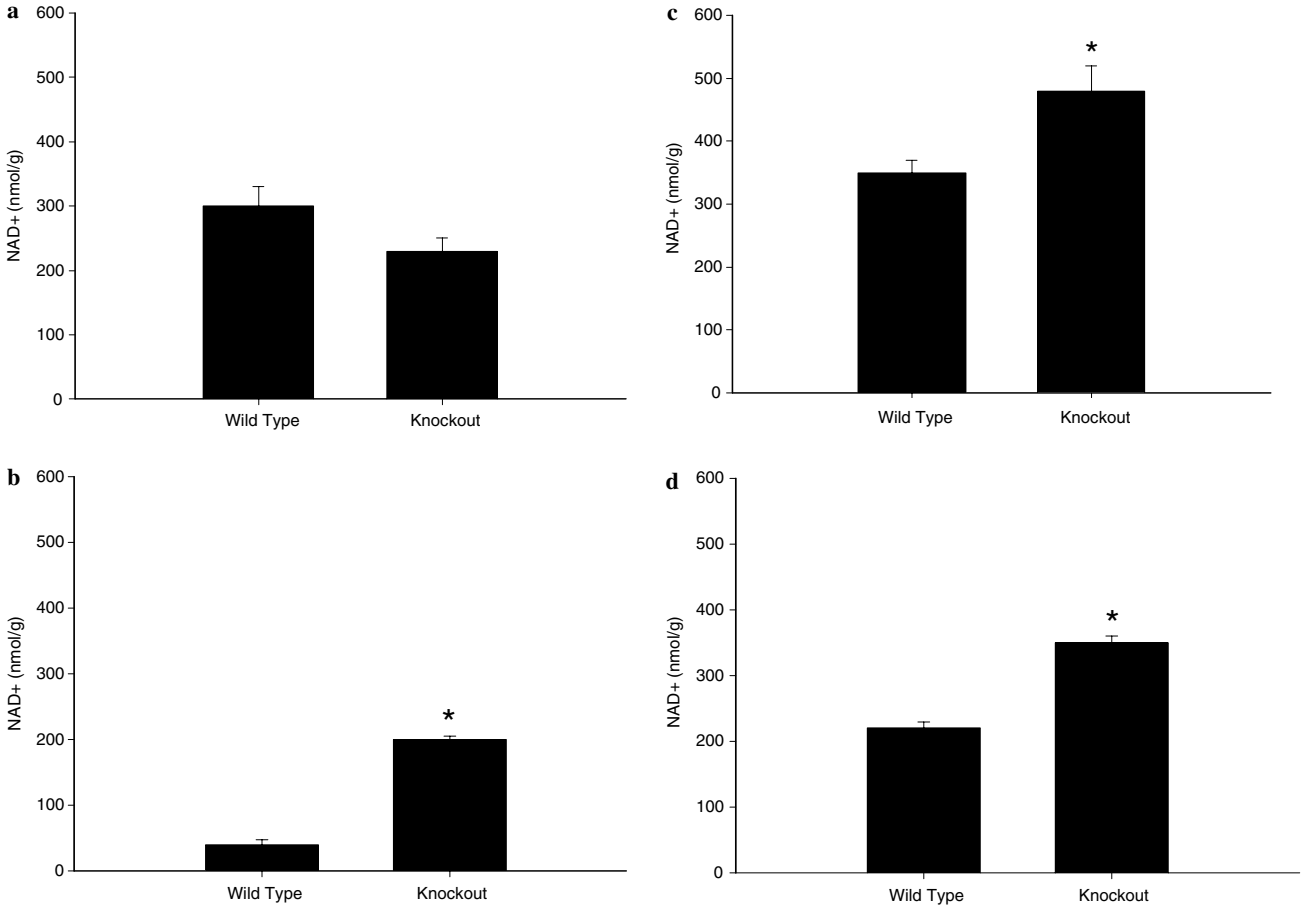

Fig. 1. (a) Heart NAD^+ in wild type and knockout mice. There was no significant difference between the two groups, $n = 5$ (2 mice per analysis). (b) Lung NAD^+ in wild type and knockout mice. Knockout mice showed significantly ($p < 0.001$) higher levels than wild type mice, $n = 5$ (2 mice per analysis). (c) Kidney NAD^+ in wild type and knockout mice. Knockout mice showed significantly ($p \leqslant 0.05$) higher levels than wild type mice, $n = 5$ (2 mice per analysis). (d) Brain NAD^+ in wild type and knockout mice. Knockout mice showed significantly ($p < 0.001$) higher levels than wild type mice, $n = 15$. * denotes a significant ($p \leqslant 0.05$) difference.

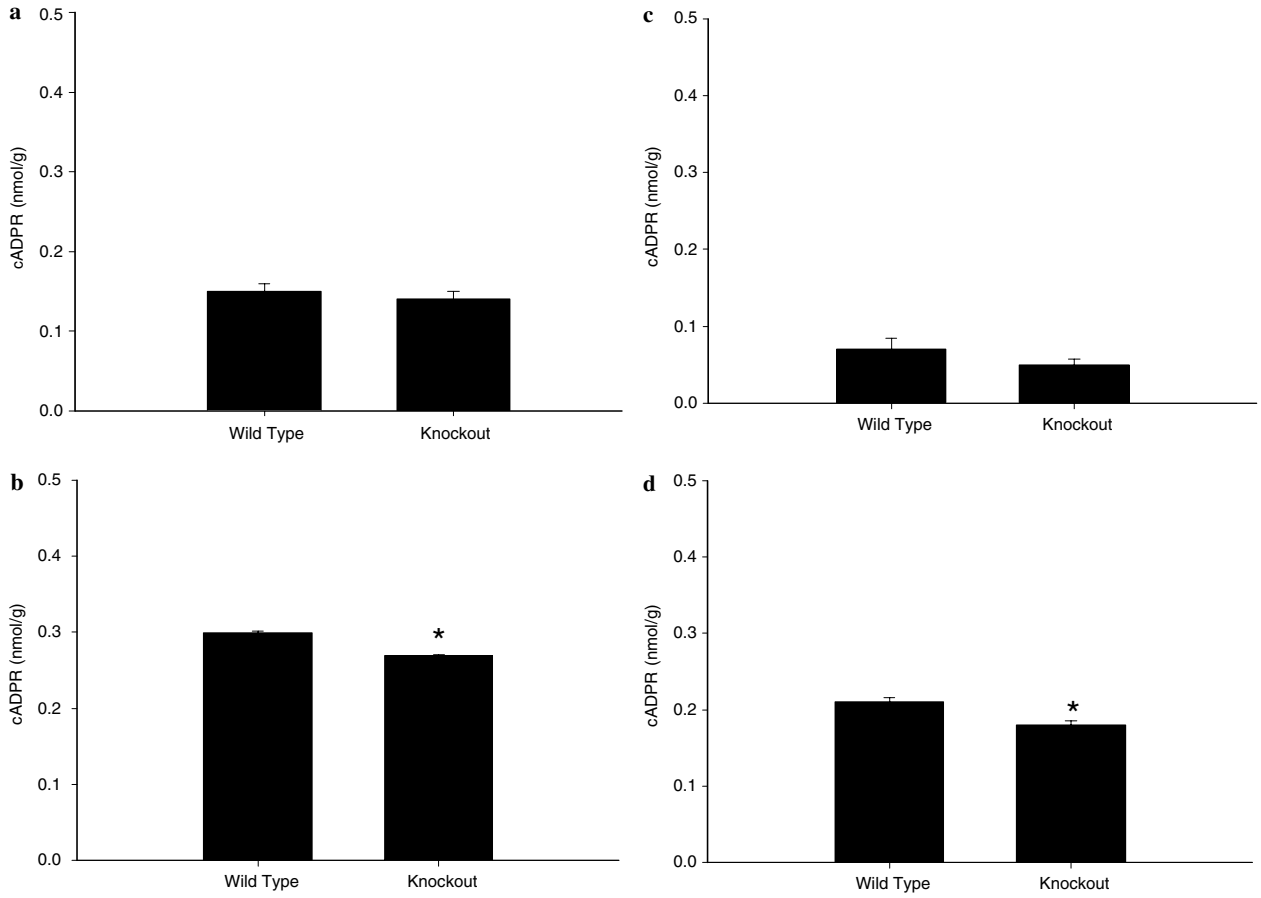

Fig. 2. (a) Heart cADPR in wild type and knockout mice. There was no significant difference between the two groups, $n = 5$ (2 mice per analysis). (b) Lung NAD^+ in wild type and knockout mice. Knockout mice showed significantly ($p < 0.001$) lower levels than wild type mice, $n = 5$ (2 mice per analysis). (c) Kidney cADPR in wild type and knockout mice. There was no significant difference between the two groups, $n = 5$ (2 mice per analysis). (d) Brain cADPR in wild type and knockout mice. Knockout mice showed significantly ($p < 0.001$) lower levels than wild type mice, $n = 15$. * denotes a significant ($p \leqslant 0.05$) difference.

and NAD^+ were measured in the heart, lungs, kidneys, and brain. All statistical analyses were performed using SPSS (Chicago, IL), version 11.5 for Windows. A significant p value was set at $\leqslant 0.05$.

Results

The results of this study are illustrated in Figs. 1 and 2. Fig. 1 shows the NAD^+ concentrations in the measured organs. Heart NAD^+(Fig. 1a) did not show a significant change due to genotype, while lung (Fig. 1b) ($p < 0.001$), kidney NAD^+ (Fig. 1c) ($p = 0.02$), and brain NAD^+ (Fig. 1d) concentrations were significantly ($p < 0.001$) increased in $Cd38^{-/-}$ as compared to wild type mice. Fig. 2 shows the cADPR concentrations in the measured organs. Heart (Fig. 2a) and kidney (Fig. 2c) cADPR did not show a significant change due to genotype, while lung (Fig. 2b) ($p < 0.001$) and brain cADPR (Fig. 2d) ($p < 0.001$) were significantly decreased in the $Cd38^{-/-}$ mice.

Discussion

This is the first study to show significant decreases in levels of brain and lung cADPR in the $Cd38^{-/-}$ mouse. Partida-Sanchez et al. (2001) previously measured levels of cADPR in these tissues and found them to be non-significantly decreased. While our levels of cADPR (with the exception of lung, discussed later) are comparable to the original report, the degree of variability in each group is reduced, which we believe is due to modifications that we have made to the fluorimetric cycling assay for cADPR. These modifications are shown to increase the recovery of cADPR, improve the functionality of the assay, and reduce between-subject variability [19]. In fact, we observed a significant reduction of brain cADPR despite a difference of only 16% between wild type and knockout mice, in contrast to the 20% non-significant reduction observed in [18] and the 18% non-significant reduction observed in [25]. The differences we measured between knockout and wild type lung cADPR were even less, with a divergence of only 10% being observed in this study, in contrast to the 57% non-significant reduction observed in [18]. However, levels of lung cADPR in this study were significantly increased in both groups relative to the original report, which is likely due to the differences in methods of organ treatment between the two studies. In Partida-Sanchez et al. (2001), mouse tissues were obtained from perfused animals, while in the present study, lung cADPR was measured in

non-perfused animals killed by CO_2 inhalation. The stress associated with the hypoxic condition causes influx of red and white blood cells into the lungs, which would still be present during nucleotide analysis and would increase cADPR levels, raising the mean levels of both groups and reducing the between-group differences, which is what we observed. The reduced variation within each group suggests that the range of cADPR is much narrower than previously thought, and that the regulatory mechanisms controlling these levels are more finely tuned. However, it is possible that since the formation and degradation of cADPR in vivo is likely to be quite rapid, measurement of static levels of cADPR in a biological system may underestimate the functional effects of CD38 deletion.

This is also the first study to measure changes in NAD^+ in the $Cd38^{-/-}$ mouse. We found that levels of NAD^+ in the brain, kidneys, and lungs increased significantly in the $Cd38^{-/-}$ mouse, with the magnitude of increase being much greater than the observed decrease in cADPR. In contrast, heart NAD^+ did not change significantly. The difference in levels of NAD^+ as compared to cADPR in all tissues is about a thousandfold, which is within the expected range [23]. In all organs, CD38 functions as both a cyclase and a hydrolase enzyme, forming ADP-ribose from hydrolysis of NAD^+ or cADPR [2]. The ratio of cyclase to hydrolase activity is low, with about 97–99% of the product of CD38 being ADP-ribose [26]. So, the loss of NAD^+ hydrolase activity might explain an increase in NAD^+ of this magnitude, and the differential activity of this enzyme would result in a much greater effect on NAD^+ increase than on cADPR reduction, which is what we observed.

The relationship between CD38, NAD^+, and cADPR has been clarified by investigations into the "topological paradox" of CD38, which concerns how CD38, with an active site that is either at the outer surface of the plasma membrane or within subcellular membrane vesicles, can regulate the formation of cADPR, which has an intracellular site of action [1]. Connexin 43 hemichannels [27] have been identified that allow passage of NAD^+ from the inside to the outside of the cell, as has bidirectional transport of cADPR through CD38 itself [28]. Intracellular NAD^+, which is found at micromolar concentrations (as compared to nanomolar concentrations extracellularly), can move down its concentration gradient through the connexin 43 channels to the ectocellular active site of CD38. CD38 can then catalyze the formation of cADPR, which passes through the central channel formed by the homodimeric structure of the protein [29]. Alternatively, cADPR can pass into the cell through nucleoside transporters [30]. The process of nucleotide transport can occur via an autocrine mechanism, with the NAD^+ and cADPR affecting the emitting cell, or a paracrine mechanism, with the nucleotides affecting cells in the vicinity of the emitting cell [29]. The changes in nucleotides reported in this study might therefore have a widespread effect on cellular function.

The observation of alterations in cADPR and NAD^+ in the $Cd38^{-/-}$ mouse illustrates the proximate relationship of CD38 with these pyridine nucleotides. While further studies are needed to delineate precisely the roles of CD38 and the CD38/cADPR system, it is possible that these may play an important role in controlling organ and cellular function, whereby changes could contribute to pathological conditions.

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