Developmental regulation of calcineurin isoforms in the rodent kidney: association with COX-2

Haiying Liu,1,2* Wenling Ye,1* Guangju Guan,2 Zheng Dong,3,4 Zhanjun Jia,1 and Tianxin Yang1

1Division of Nephrology, University of Utah and Veterans Affairs Medical Center, Salt Lake City, Utah; 2Department of Nephrology, Shandong University Medical School, Jinan, Shandong Province, Peoples Republic of China; 3Cellular Biology and Anatomy, Medical College of Georgia, Augusta; and 4Medical Research Service, Veterans Affairs Medical Center, Augusta, Georgia

Submitted 1 August 2007; accepted in final form 5 September 2007

Liu H, Ye W, Guan G, Dong Z, Jia Z, Yang T. Developmental regulation of calcineurin isoforms in the rodent kidney: association with COX-2. Am J Physiol Renal Physiol 293: F1898–F1904, 2007. First published September 19, 2007; doi:10.1152/ajprenal.00360.2007.—Calcineurin (Cn)-Aα-deficient mice develop abnormalities of postnatal kidney development, similar to that of cyclooxygenase (COX)-2-deficient mice. The present study was undertaken to examine expression and regulation of Cn isoforms in the developing kidney during the postnatal period and further characterize the relationship between Cn and COX-2. The protein expressions of all three Cn isoforms, including Cn-Aα, -Aβ, and -B, as determined by immunoblotting, increased in parallel in the first postnatal week and declined gradually with age. Renal Cn-Aα and -Aβ mRNA expressions were both developmentally regulated in the same fashion as their protein expressions, whereas renal Cn-B1 mRNA was not obviously induced in the first postnatal week. Immunohistochemistry demonstrated colocalization of Cn-Aα, Cn-Aβ, and COX-2 in the same cells of thick ascending limb and macula densa. Administration with cyclosporine A (2.5 mg·kg−1·day−1) during the postnatal period remarkably suppressed renal COX-2 expression as assessed by both immunoblotting and immunohistochemistry. Deletion of Cn-Aα but not Cn-Aβ in mice significantly reduced renal COX-2 expression at the postnatal period. Together, these data suggest that renal Cn isoforms are subject to normal developmental regulation and they may play a role in postnatal kidney development via interaction with COX-2.

Cyclooxygenase-2: postnatal kidney development; cyclosporine A

Calcineurin (Cn), also called protein phosphatase 2B, is a heterodimeric Ca2+/calmodulin-dependent serine/threonine protein phosphatase composed of two subunits, the catalytic subunit Cn-A (59–62 kDa) and the regulatory subunit Cn-B (19 kDa; see Ref. 23). Cn-A exists in three distinct forms [α, β, and γ isoforms (Cn-Aα, Cn-Aβ, and Cn-Aγ)]; Cn-B exists in two forms (Cn-B1 and Cn-B2). Cn-Aγ expression is restricted to testis and brain, and Cn-B2 is testis specific, whereas the other isoforms of Cn are quite ubiquitously expressed in a wide variety of tissues (23). Cn is a key component of the calcium-initiated signal transduction pathway involved in a wide spectrum of cellular function. The importance of this pathway in the kidney is highlighted by the well-recognized adverse renal effect associated with Cn inhibitors cyclosporine A (CsA) and FK-506 (7, 18, 32, 33). Phenotypic analysis of mice with systemic deletion of Cn-Aα and -Aβ reveals distinct function of the two A-isoforms in the kidney; Cn-Aβ knockout (KO) mice have virtually normal renal phenotype, whereas Cn-Aα KO mice develop abnormalities of postnatal kidney development and chronic renal failure (12). Systemic deletion of Cn-B produces a lethal phenotype, and conditional deletion of this Cn isoform in the urogenital tract induces defective pyeloureteral peristalsis and obstructive nephropathy that ultimately lead to renal failure (4). Compared with the detailed information about the role of Cn isoforms in the development of the kidney, the expression and regulation of the individual Cn isoforms in the developing kidney are poorly characterized.

Systemic deletion of the cyclooxygenase (COX)-2 gene by homologous recombination is consistently associated with abnormal postnatal kidney development, characterized by dysplasia of the nephrogenic zone that contains increasing numbers of immature glomeruli and atrophied renal tubules (19, 20). The phenomenon was later recapitulated by pharmacological administration of pregnant rats with the COX-2 inhibitor SC-58236 (14), which led to renal histological abnormalities at the postnatal but not prenatal period. In support of the role of COX-2 in postnatal nephrogenesis, renal COX-2 expression is developmentally regulated with a transient induction around postnatal day (PND) 7 that declines rapidly with age and reaches the lowest level in adults (16, 21, 28, 31). This raises an important question as to what factors control the physiological regulation of renal COX-2 expression during the postnatal period.

Prompted by the above observations, the present study was undertaken to characterize expression patterns of Cn isoforms in the developing kidney during the postnatal period and to further characterize the relationship between Cn and COX-2. We provide evidence that all three Cn isoforms tested are subject to normal developmental regulation, with a peak induction in the first postnatal week. Among them, Cn-Aα and -Aβ colocalize with COX-2 to the cortical thick ascending limb (cTAL) of Henle’s loop and macula densa, whereas Cn-B is mostly restricted to the thin limb of the loop. We further demonstrate that the postnatal induction of renal COX-2 expression is under the control of Cn.

METHODS

Animals. Three mouse lines lacking Cn-Aα, Cn-Aβ, and COX-2 were originally generated by Zhang et al. (30), Bueno et al. (2), and Dinchulk et al. (8), respectively. Theses mouse lines were maintained
at the Animal Facility of the University of Utah. The Cn-Aα and Cn-Aβ null mutations were propagated on a mixed genetic back-
ground of 129/Sv and C57/BL6, whereas COX-2 KO mice had a
congenic background (129/Sv) generated by backcrossing for more
than 10 generations (29). Genomic DNA was isolated from a short
piece of tail taken at the time of weaning and genotyping of COX-2-
deficient (Cn-Aα/H9250), Cn-Aα/H9251/H11002, and Cn-Aα/H9252/H11002
mice, as previously de-
scribed (2, 29, 30). Female Sprague-Dawley rats with litters were
from Harlan Laboratories. All animal procedures were approved by
the University of Utah Institutional Animal Care and Use Committee.

Procedures for drug treatments of young rats. Starting from PND1,
the rat pups received daily subcutaneous injections of vehicle (saline)
or the Cn inhibitor cyclosporine at 2.5 mg·kg⁻¹·day⁻¹ (Bedford
Laboratories, Bedford, OH) until PND10. During the 10-day treat-
ment period, the female rats were paced on standard rat chow and had
free access to tap water. At the end of experiments, under general
anesthesia, kidneys were harvested and processed for real-time RT-
PCR, immunoblotting, and immunohistochemistry.

Morphological studies. Kidneys were hemisectioned and fixed by
direct immersion in 3% paraformaldehyde for 16 h. Following embed-
ning in paraffin, 5-μm sections were prepared and stained with
hematoxylin and eosin and analyzed with a light microscope.

Immunoblotting. Lysates of the kidney were stored at −80°C until
assayed. Protein concentrations were determined using Coomassie
reagent. An equal amount of the whole tissue protein (60 μg) was
denatured at 100°C for 10 min, separated by SDS-PAGE, and trans-
ferred to nitrocellulose membranes. The blots were blocked overnight

Fig. 1. Immunoblotting analysis of calcineurin (Cn) isoform protein expres-
sion in the kidney of Sprague-Dawley rats at the indicated ages. A: represent-
ative blots for Cn-Aα, Cn-Aβ, and Cn-B. β-Actin serves as a loading control.
B: densitometric analysis of Cn-Aα protein. C: densitometric analysis of
Cn-Aβ protein. D: densitometric analysis of Cn-B protein. Densitometric data
were generated from one representative of 2–3 separate experiments; n = 3
mice in each group. PND, postnatal day. *P < 0.01 vs. PND1; #P < 0.05 vs.
PND1.

Fig. 2. Real-time RT-PCR analysis of mRNA expression of Cn-Aα (A),
Cn-Aβ (B), and Cn-B1 (C) in the kidney of Sprague-Dawley rats at the
indicated ages. Relative abundance of individual Cn isoforms was normalized
by glyceraldehyde-3-phosphate dehydrogenase; n = 6 in each group. *P <
0.05 vs. PND1.
with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation for 1 h with rabbit polyclonal antibodies against Cn-Aα, Cn-Aβ, and Cn-B (1:500 dilution for all Cn antibodies; Santa Cruz) or monoclonal antibody against β-actin (1:1,000 dilution; Sigma). The blots were washed with TBS followed by incubation with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody for Cn isoforms or goat anti-murine HRP-conjugated secondary antibody for β-actin. Immune complexes were detected using enhanced chemiluminescence methods. The immunoreactive bands were quantified using the Gel and Graph Digitizing System (Silk Scientific).

**Real-time RT-PCR.** Total RNA was isolated using Tri-Zol (Invitrogen), and cDNA was synthesized using SuperScript first strand (Invitrogen). The sequence of oligonucleotides used for real-time PCR is listed as follows: Cn-Aα sense 5'-AAGCGGATCCCAGT-3', Cn-Aα antisense 5'-AACGCCAGACCTTCCTCAG-3'; Cn-Aβ sense 5'-TGATTTCCATTTGTGGA-3', Cn-Aβ antisense 5'-CTTTACTTTTGCCAAT-3'; Cn-B1 sense 5'-CTGGTGTTACATTTGTTGGA-3', Cn-B1 antisense 5'-GCTTTCACTTTCTTCCTGA-3'. Real-time RT-PCR was performed using Sybergreen and the ABI Prism 7900 Sequence Detection System. The amplification was carried out for 40 cycles with conditions of 15 s denaturation at 95°C, 45 s annealing, and extension at 60°C. Real-time RT-PCR of COX-2 was performed as previously described (22).

**Immunohistochemistry.** For comparison of localization of Cn isoforms and COX-2, immunohistochemistry was performed on fixed kidney sections as previously described (31). Briefly, kidneys were perfused in situ with saline containing 0.02% sodium nitrite and heparin (10 U/ml) and fixed by perfusion with 3.7% formaldehyde, 1.4% lysine, 0.01 M sodium metaperiodate, 0.04 M sodium phosphate, and 1% acetic acid. The kidneys were then dehydrated with a graded series of ethanol and embedded in paraffin. Sections were mounted on glass slides and immunostained with the polyclonal goat anti-murine Cn-Aα, Cn-Aβ, and Cn-B antiserum (1:50 dilution). The avidin-biotin complex kit (Santa Cruz) was used to localize the primary IgGs with a chromogen of oxidized diaminobenzidine. For assessment of the suppression of renal COX-2 expression by CsA treatment, immunostaining of COX-2 was performed on cryostat kidney sections prepared from the pups treated for 10 days with vehicle or CsA. The sections were incubated in 0.5% Triton X-100/PBS for 30 min. After rinsing in PBS, nonspecific protein-binding sites were blocked by a 2-h incubation with 5% dry milk. The polyclonal antibody against COX-2 (Cayman) was applied in an 1:100 dilution in 5% dry milk overnight at room temperature. After rinsing in PBS, signals were detected with a Cy2-labeled secondary antibody and viewed in an Olympus IMT-2 microscope with fluorescence module.

**Statistical analysis.** Values shown represent means ± SE. Analysis of densitometry and real-time RT-PCR data from Sprague-Dawley rats at various ages was performed using one-way ANOVA followed by a Bonferroni posttest. Analysis of differences between two

---

**Fig. 3.** Colocalization of Cn-Aα (A), Cn-Aβ (B), and cyclooxygenase (COX)-2 (C) to the same cells of cortical thick ascending limb (cTAL) and macula densa in 20-day-old Sprague-Dawley rats. Immunohistochemistry was performed on consecutive sections prepared from perfusion-fixed kidney samples. Cn-B immunoreactivity was detected in the renal medulla (E at ×25) but not in renal cortex (D at ×25). At higher magnification (F at ×100), Cn-B immunoreactivity was found in the thin limb of Henle’s loop (arrows), and collecting duct (CD) was stained negative.
groups (KO vs. wild type) was performed using an unpaired t-test. P value < 0.05 was considered significant.

RESULTS

Regulation of renal Cn isoforms during the postnatal period.

Figure 1 shows protein expressions of Cn-Aα, -Aβ, and -B in the kidney of Sprague-Dawley rats from newborn through PND28, as determined by immunoblotting, and β-actin was used as a loading control. Cn-Aα and -Aβ were both detected as ~60-kDa bands and Cn-B as ~20-kDa bands. Overall, renal protein expressions of the three Cn isoform exhibited a similar pattern of developmental regulation; all isoforms were detectable at newborn, peaked at PND7, and declined gradually with age. However, the magnitude of induction at PND7 appeared obvious (Fig. 2, A and B). In contrast, the induction of Cn-B1 mRNA in the first postnatal week was not obvious (Fig. 2B). The degree of induction of mRNA expression in the first postnatal week (PND7 vs. PND1) was 2.0-fold for Cn-Aα (n = 6 in each group, P < 0.01), ~2-fold for Cn-Aβ (n = 3, P < 0.05), and ~3.5-fold induction for Cn-B (n = 3, P < 0.01).

Renal mRNA expressions of Cn-Aα, -Aβ, and -B in the kidney of Sprague-Dawley rats from newborn through PND28 were quantified by real-time RT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Consistent with the protein expression pattern, renal Cn-Aα and -Aβ mRNA expressions were both developmentally regulated, with a peak induction in the first postnatal week, followed by a gradual reduction with age (Fig. 2A and B). In contrast, the induction of Cn-B1 mRNA in the first postnatal week was not obvious (Fig. 2C). The degree of induction of mRNA expression in the first postnatal week (PND7 vs. PND1) was 2.0-fold for Cn-Aα (n = 6 in each group, P < 0.05), 2.7-fold for Cn-Aβ (n = 6 in each group, P < 0.05), and 1.6-fold for Cn-B1 (n = 6 in each group, P > 0.05).

Immunohistochemistry was performed to determine cellular localization of Cn-Aα, -Aβ, and -B compared with COX-2 in the kidney of 20-day-old rats. This age was chosen due to the requirement of animal size for performance of perfusion fixation of the kidney. As expected, COX-2 immunoreactivity (ir) was detected in the cTAL and macula densa (Fig. 3C). Strikingly, Cn-Aα-ir and Cn-Aβ-ir were colocalized with COX-2-ir to the same epithelial cells (Fig. 3, A and B). However, Cn-B-ir exhibited a distinct pattern with restricted activity in the thin limb of Henle’s loop in the renal medulla (Fig. 3, D-F).

Effects of Cn inhibition on COX-2 expression in the developing kidney. To determine whether COX-2 expression in the developing kidney was under the control of Cn, inhibition of Cn was achieved by administration of the Cn inhibitor CsA at the early postnatal period and by the use of Cn-Aα KO mice. Newborn Sprague-Dawley rats received daily subcutaneous injections of vehicle and the Cn inhibitor CsA (2.5 mg·kg⁻¹·day⁻¹) for 10 days, and renal COX-2 expression was assessed using immunoblotting and immunostaining. Following the 10-day CsA treatment, the total abundance of renal COX-2 was remarkably diminished (Fig. 4A, left). In line with these data, immunostaining revealed intense COX-2 labeling in cTAL in the vehicle group contrasting the almost complete disappearance of the labeling in the CsA group (Fig. 4B). To minimize the confounding influence from the chronic CsA treatment, the effect of a single dose of CsA on COX-2 expression was examined. As shown in Fig. 4A, right, the single CsA treatment also exhibited a significant inhibitory effect on COX-2 expression, suggesting a primary rather than a secondary effect of CsA.

As previously described (12), Cn-Aα⁻/⁻ mice had reduced survival and were infertile, a majority of the Cn-Aα⁻/⁻ mice died around the weaning period, and only ~10–15% of them lived till adulthood. Figure 5 shows renal histology from mice lacking Cn-Aα, Cn-Aβ, and COX-2 at the indicated ages. Although no immature glomeruli were found in the superficial cortex (nephrogenic zone) of wild-type mice, a persistent presence of immature glomeruli was found in the superficial cortex of 28-day-old Cn-Aα⁻/⁻ and COX-2⁻/⁻ mice. At PND7, Cn-Aα⁻/⁻ kidney exhibited roughly normal histology. Cn-Aβ⁻/⁻ were grossly indistinguishable from wild-type mice and had normal survival. Renal histology of Cn-Aβ⁻/⁻ mice examined at PND28 did not exhibit significant changes compared with wild-type mice. We determined COX-2 expression in the kidney of 7-day-old Cn-Aα⁻/⁻ and Cn-Aβ⁻/⁻ mice.
using real-time RT-PCR. COX-2 expression was reduced significantly in Cn-Aα−/− [n = 11 for Cn-Aα-sufficient (+/+)] and n = 9 for Cn-Aα−/−, P < 0.01] but not Cn-Aβ−/− (n = 4 for Cn-Aβ+/+ and n = 5 for Cn-Aβ−/−, P < 0.01) mice.

DISCUSSION

Kidney development is a complex process involving early inductive interaction between the ureteric bud and metanephric mesenchyme, and later maturation of nephrogenic zone, which continues after birth (1, 9–11, 15, 17, 24, 27). Many of the transcription factors and signaling pathway in the early phase of kidney development have been elucidated, whereas the molecular pathway controlling the later phase of kidney development at the postnatal period is poorly understood. Emerging evidence indicates involvement of both Cn and COX-2 in postnatal nephrogenesis. The present study has made the following two major contributions: 1) characterization of Cn isoform expression and regulation in the kidney during the postnatal period and 2) characterization of the relationship between Cn and COX-2 in the developing kidney.

Cn is a heterodimeric Ca²⁺/calmodulin-dependent serine/threonine protein phosphatase, playing a critical role in development and cellular function, primarily via activation of NFAT that controls target gene transcription. A specific role of Cn in kidney development was examined by Tendron et al. (25) who administered CsA to pregnant rabbits and demonstrated reduced nephron number and impairment of the nephrogenic zone. Recently, Gooch et al. (12) analyzed the renal phenotype of mice lacking the respective Cn-A isoforms and have assigned an important role of Cn-Aα in postnatal kidney development. Consistent with the results of Gooch et al., we demonstrated that, among the three Cn isoforms assayed, Cn-Aα exhibited the greatest dynamic alteration, with approximately fivefold induction in protein expression in the first postnatal week that progressively declined with age. Although less pronounced compared with Cn-Aα, the two other Cn isoforms, Cn-Aβ and Cn-B, exhibited a similar pattern of developmental regulation. The normal renal histology in Cn-Aβ-deficient mice does not support a role of Cn-Aβ in postnatal kidney development. The knowledge about Cn-B in kidney development is scarce. Therefore, the implication of developmental regulation of the later two Cn isoforms still remains elusive.

Cn-B is a regulatory subunit critically important for determination of Cn activity. However, to our knowledge, there are no prior studies examining expression or regulation of Cn-B in the kidney. We found that Cn-B in the kidney is subject to

Fig. 5. A: renal histology in mice lacking Cn-Aα, Cn-Aβ, and COX-2 at the indicated ages. Arrows indicate similar lesions (increased numbers of small glomeruli in the subcapsular region) in Cn-Aα−/− and COX-2−/− mice that were not seen in the age-matched wild-type mice. Cn-Aβ−/− mice at PND28 had normal renal histology. B: real-time RT-PCR detection of COX-2 mRNA in the kidney of 7-day-old Cn-Aα−/− and Cn-Aβ−/− mice compared with respective wild-type controls; n = 4–11 in each group.
developmental regulation, in parallel with other Cn isoforms, with an exception that the age-dependent change in Cn-B expression occurs only at the protein but not mRNA level, suggesting a posttranscriptional mechanism. We further localized Cn-B-ir to the thin limb of Henlé’s loop in the developing kidney. Recently, Chang et al. (4) created site-specific deletion of Cn-B1 in the mesenchyme of the developing urinary tract (flooded Cn-B1 crossed with PAX3Cre mice) that leads to congenital obstructive nephropathy, suggesting an essential role of Cn-B1 in the development of the urinary tract system. The same Cre-loxP approach may be necessary to address the function of Cn-B in kidney development.

The above information adds to the knowledge of Cn isoform expression and regulation in the kidney. There are a limited number of reports on distribution of Cn isoforms in the rat kidney under basal and disease states. Tumlin et al. (26) examined Cn activity in isolated nephron segments and demonstrated that enzyme activity is 10-fold higher in the S2 segments of the proximal tubule than that in cortical collecting duct, cortical connecting tubule, or medullary thick ascending limb (mTAL). In parallel with Cn activity, Cn-A protein expression in the S2 segments as assessed by immunoblotting is greater than that of other segments, whereas the highest amount of Cn-Aβ protein is found in mTAL (26). Buttini et al. (3) examined renal Cn isoform mRNA expression using in situ hybridization and found predominant mRNA expression of both A isoforms in the renal medulla, which did not entirely correspond to the results of Tumlin et al. Our results are generally consistent with those of Gooch et al. (12) showing a parallel induction of Cn-Ao and Aβ in cTAL and macula densa in the diabetic kidney. There is an exception, however, that the induction of Cn-Ao expression was also found in glomeruli and the collecting duct after prolonged diabetes (12), whereas these segments were stained negative for all three Cn isoforms in our study.

Mice deficient in Cn-Ao and COX-2 developed a similar phenotype characterized by abnormal postnatal kidney development, including dysplasia of the nephrogenic zone that contains increasing numbers of immature glomeruli and atrophied renal tubules (12, 19, 20). The phenotypic similarity between the two mutants presents an intriguing possibility that the two enzymes may act in the same pathway during postnatal nephrogenesis. In support of this possibility, Cn inhibitors are reported to inhibit COX-2 expression in the kidney of adult Sprague-Dawley rats (13) and in several cell culture systems (5, 6). Furthermore, COX-2 promoter contains NFAT-responsive elements (13). However, no prior studies specifically examined interaction of the two enzymes in the developing kidney. The present study provides compelling evidence supporting such interaction. First, gene expression of Cn-Ao as well as Cn-Aβ and Cn-B was all induced in the first postnatal week, which coincides with the time frame of COX-2 induction (16, 21, 28, 31). Furthermore, the two A isoforms colocalized with COX-2 to the same cells of cTAL and macula densa, presenting strong anatomical evidence for the unique association between Cn and COX-2. Second, COX-2 expression in the developing kidney of rats was inhibited by postnatal administration with CsA. The reduction of COX-2 expression was even achieved by a single dose of CsA, which is unlikely associated with confounding influences seen with chronic CsA treatment. Finally, renal COX-2 expression was reduced in 7-day-old Cn-Ao KO but not Cn-Aβ KO mice compared with wild-type controls. These observations suggest that the postnatal induction of renal COX-2 expression may be under the control of Cn and that this interaction may be essential for postnatal kidney development. The mechanism concerning Cn regulation of COX-2 still remains uninvestigated. Because COX-2 promoter contains NFAT-responsive elements, it is possible that Cn may directly regulate COX-2 gene transcription. It is also possible that Cn may affect COX-2 expression in an indirect manner. For example, the changes in COX-2 expression might be secondary to changes in sodium handling in the thick ascending limb following CsA treatment. Cn inhibition has been reported to inhibit Na-K-ATPase activity isolated in nephron segments in vitro (26).

In summary, the present study demonstrated that renal expression of all three Cn isoforms is subject to developmental regulation with a parallel induction in the first postnatal week that coincides with the time frame of postnatal induction of COX-2 expression. By immunohistochemistry, Cn-Ao and Cn-Aβ colocalize with COX-2 to the same cells of cTAL and macula densa. Inhibition of Cn during the postnatal period by administration of CsA and genetic deletion of Cn-Ao significantly suppressed COX-2 expression. These findings suggest that interaction of Cn and COX-2 might be required for postnatal nephrogenesis.

ACKNOWLEDGMENTS

We thank Jeffery D. Molkentin (Children’s Hospital Medical Center, Cincinnati, OH) for providing breeder pairs of Cn-Aβ KO mice. Jonathan Seidlin (Brandeis Medical School) for providing breeder pairs of Cn-Ao KO mice, and Hui Zhang, Kenneth Heal, and Noel G. Carlson (University of Utah and Salt Lake Veterans Affairs Medical Center, Salt Lake City, Utah) and Mingzhii Zhang (Vanderbilt University) for technical assistance.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant RO-1 HL-079453 (to T. Yang). H. Liu was an exchange graduate student and was partially supported by Shandong University and by a grant from the National Kidney Foundation of Utah and Idaho.

REFERENCES

8. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffe BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Gorry SA,


